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The role of oestrogens and antibiotics on the development of cancer

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THE ROLE OF OESTROGENS AND ANTIBIOTICS ON THE DEVELOPMENT OF CANCER
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By

Johanna Simin

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To Sebastian, Mathilda, and Goran

“Medicine is a science of uncertainty and an art of probability.”

- Sir William Osler (1849 – 1919)

SHORT POPULAR SCIENCE SUMMARY

This thesis aimed to investigate different long-term effects of menopausal hormone therapy (MHT) and oral antibiotic treatment – both common pharmacological treatments, which either include oestrogens or may alter the metabolism of oestrogens. Three of the included publications were based on extensive nationwide and population-based cohort studies evaluating the risk of cancer among MHT users, including virtually all Swedish women exposed to MHT. Menopausal hormone therapy is the primary treatment for vasomotor symptoms of menopause, which are experienced by 50 – 80% of women worldwide. One of the publications focused on oral antibiotic treatment exploring the posed association with colorectal cancer risk since the widespread use of common antibiotics and the increasing burden of cancer highlights the need to investigate all potential risk factors.

We have shown that the cancer risk associated with systemic MHT is seemingly overestimated, given the net effect of menopausal hormones on the cancer risk was only slight. The excess cancer risk was mainly linked with an increased risk of breast and other central female reproductive organ cancers, particularly among women who initiated treatment at an older age. In contrast, we found a lower risk of gastrointestinal tract cancers among MHT users, and our data indicate that prediagnostic use of unopposed oestrogens-only may even improve survival from colorectal cancer. These findings could further support the chemopreventive role of oestrogens associated with sex hormone-related cancers.

Whereas we have seen that the number of MHT prescriptions has remained rather stable during the past decade, the number of MHT prescriptions dropped by over 30% in Sweden after the millennium following trial results indicating higher breast cancer risk. This drop in the MHT prescriptions can partly be explained by fear of the MHT use associated carcinogenic effects. However, our results have shown that menopausal hormones do not appear to increase the cancer risk when initiated near to menopausal onset among otherwise healthy women without contraindications or an increased risk of breast, ovarian or endometrial cancer.

Furthermore, we found an association between exposure to commonly prescribed antibiotics and excess colorectal cancer risk, linked with especially broad-spectrum antibiotics. However, evidence for the dose-response relationship was weak, and the shown association may not be causal. Nonetheless, together with antibiotics' widespread use, these results warrant more strict antibiotic stewardship.

ABSTRACT

Despite advances in cancer treatment and surgery, cancer-related mortality remains the leading cause of death globally, harvesting over ten million lives annually. While the risk factors are both hormonal and non-hormonal, common prescription drugs' long-term effects are understudied and likely even underestimated. Particularly drugs including oestrogens or those altering the metabolism of oestrogens may promote carcinogenesis of oestrogen receptor sensitive cancers and possibly even lower the risk of sex hormone-associated cancers. Two common examples of such prescription drugs are systemic menopausal hormone therapy (MHT) and oral antibiotic treatment.

Historically, preventive measures have been the key factor in our unending battle against cancer's burden. This doctoral thesis within clinical epidemiology was conducted to evaluate the association between exposure to contemporary MHT use and cancer risk (Studies I and II); and colorectal cancer mortality (Study IV). These associations were investigated on a population level, longitudinally, based on nationwide and population-based cohort studies, taking advantage of the unique Swedish prescription feature, including various MHT treatment options. Notably, these large-scale cohort studies are feasible to conduct only in a few other countries than Sweden, underscoring the importance of valid evidence from methodologically well-grounded studies with complete and reliable data sources. Furthermore, Study III aimed to clarify the posed association between exposure to oral antibiotic treatment and colorectal cancer risk based on a systematic review and dose-response meta-analysis.

For Studies I, II, and IV, data were retrieved from the Swedish Prescribed Drug Registry, Swedish Cancer Registry, Causes of Death Registry, Patient Registry, and the Total Population Registry (Study I only). **Study I** evaluated the net effect of systemic MHT use on cancer risk; and 16 different cancer types. The findings suggested an association of menopausal hormones with a slightly (9%) elevated cancer risk compared with the background population of the same calendar period and age. The observed excess risk of cancer was mainly associated with female reproductive tract cancers, yet it was almost counterbalanced by the reduced risk of gastrointestinal tract cancers. However, the cancer risk varied between the different MHT treatment options and age at treatment initiation. **Study II** was a population-based matched cohort study evaluating the link between ever-use of MHT and ovarian cancer risk. Whereas ovarian cancer is rare, it is the most lethal of gynaecological cancers, owing primarily to late detection, underscoring the need for preventive measures. The results suggested that the excess ovarian cancer risk was linked particularly with

oestrogen combined progestin use, whilst no increased risk was shown among oestrogen-only users. Furthermore, cutaneous preparations indicated a possibly less prominent risk than oral MHT. **Study IV** was a nationwide cohort study investigating whether prediagnostic exposure to MHT treatment could influence colorectal cancer-specific or all-cause mortality risk among women diagnosed with colorectal cancer. The results suggested particularly past users of oestrogen-only therapy having a possibly better colorectal cancer survival than women diagnosed with colorectal cancer who did not receive menopausal hormones during the study period.

Study III aimed to explore the posed link between oral antibiotic use with colorectal cancer risk. At the time being, the present study was the first systematic review and meta-analysis assessing the risk pattern's shape, considering possible deviation from linearity. The results indicated modestly increased colorectal cancer risk, and the association appeared to be related particularly with the use of broad-spectrum antibiotics. However, the found dose-response relationship was weak, with similar risk patterns within the colorectal continuum.

In conclusion, the risk of cancer associated with MHT treatment varies between the different treatment options and ages. The excess risk is seemingly related to female reproductive tract organ cancers, while the risk of gastrointestinal tract cancers appears to be lower, and prediagnostic use of MHT might even improve survival from colorectal cancer. Overall, MHT does not seem to increase cancer risk if the treatment is initiated near to menopausal onset among women without contraindications or high risk of breast, ovarian or endometrial cancer. The found association of oral antibiotic treatment with colorectal cancer raises further questions, and notably, the here shown association may not be causal.

LIST OF SCIENTIFIC PAPERS

I. **SIMIN J**, Tamimi R, Lagergren J, Adami H. O, Brusselaers N.
Menopausal hormone therapy and cancer risk: An overestimated risk?
European Journal of Cancer 2017; **84**: 60-68.

II. **SIMIN J**, Tamimi R. M, Callens S, Engstrand L, Brusselaers N.
Menopausal hormone therapy treatment options and ovarian cancer risk: A Swedish prospective population-based matched-cohort study.
International Journal of Cancer 2020; **147**(1): 33-44.

III. **SIMIN J**, Fornes R, Liu Q, Olsen R. S, Callens S, Engstrand L, Brusselaers N.
Antibiotic use and risk of colorectal cancer: A systematic review and dose-response meta-analysis.
British Journal of cancer 2020; **123**(12): 1825-1832.

IV. **SIMIN J**, Liu Q, Wang X, Fall K, Williams C, Callens S, Engstrand L, Brusselaers N.
Prediagnostic use of oestrogen-only therapy is associated with improved colorectal cancer survival in menopausal women: A Swedish population-based cohort study.
Acta Oncologica 2021. (1651-226X (Electronic)).
Doi: 10.1080/0284186X.2021.1909747.

LIST OF SELECTED SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. **SIMIN J**, Tamimi R. M, Engstrand L, Callens S, Brusselaers N.
Antibiotic use and the risk of breast cancer: A systematic review and dose-response meta-analysis
Pharmacological Research 2020; **160**: 105072.
Doi: 10.1016/j.phrs.2020.105072

II. LIU Q, **Simin J**, Debelius J, Fall K, Sadr-Azodi O, Engstrand L, Williams C, Brusselaers N.
Different menopausal hormone therapies and risk of colorectal cancer: A Swedish matched-cohort study.
Alimentary Pharmacology & Therapeutics. LID - 10.1111/apt.16362 [doi]. (1365-2036 (Electronic)).

III. KHODIR H, Fornes R, **Simin J**, Stål P, Duberg A-S, Brusselaers N, Aleman S.
Risk of Hepatocellular Carcinoma in Hepatitis B and D Virus Co-Infected Patients: A Systematic Review and Meta-analysis of Longitudinal Studies.
Revision submitted in April 2021.

CONTENTS

LIST OF ABBREVIATIONS

CI	Confidence interval
CRC	Colorectal cancer
E-MHT	Oestrogen-only therapy
EP-MHT	Oestrogen combined progestin therapy
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
ES	Effect size
HR	Hazard ratio
ICD	International Classification of Diseases
MHT	Menopausal hormone therapy
OR	Odds ratio
RCT	Randomised controlled trial
RR	Relative risk
SIR	Standardised incidence ratio
VMS	Vasomotor symptoms

1 INTRODUCTION

The long-term effects of common prescription drugs are rarely studied and likely underestimated. However, the overall high burden of cancer and a large proportion of prescription drug users highlights the importance of considering the use of prescription drugs as a possible risk factor for cancer, even if some drugs might seem to have chemopreventive properties, especially for sex hormone-associated cancers.(1-4) In Sweden, nearly seven million individuals were exposed to at least one common prescription drug in 2017 alone (±85% of the total population), and virtually the entire population is exposed to some drugs during their lifetime.(5) Despite the improved antibiotic stewardship, penicillin V remains among the most commonly prescribed drugs in Sweden.(5) Furthermore, at least one-third of Swedish women are estimated to require menopausal hormone therapy (MHT) to alleviate vasomotor symptoms (VMS), which may diminish the quality of life if untreated.(6)

Whereas it is becoming established that MHT use increases the risk of particularly breast cancer,(7-10) some evidence advocates oestrogens reducing the risk of colorectal cancer (CRC) similarly to other chemopreventive agents like non-steroidal anti-inflammatory drugs and aspirin.(3, 4, 11, 12) Although oestrogens possess carcinogenic properties, this could potentially support the preventive role of oestrogens, particularly for sex hormone-related cancers,(3, 13-15) or it could imply a part of inflammation and infection in carcinogenesis.(16, 17) Furthermore, commonly prescribed antibiotics have been linked with elevated colorectal cancer risk and other even more distal cancers.(16, 18, 19) One possible explanation could be antibiotic use following changes in the gut microbiome composition and alterations in oestrogen's metabolism,(20-22) - a hypothesis that is further supported by the shown microbiome changes among individuals who have been diagnosed with colorectal cancer.(16)

This doctoral thesis within clinical epidemiology aims to investigate the role of two common prescription drugs on the risk of cancer and cause-specific mortality, evaluated on a population level. Specifically, this thesis aims to evaluate the association of systemic MHT use on cancer risk and colorectal cancer mortality; and clarify the posed association between oral antibiotic treatment and colorectal cancer risk. The specific knowledge gaps and novelty of the studies included in this thesis are presented in **Table 1**. The obtained results may provide valuable information to aid in clinical risk assessment, identifying MHT treatment options, which may include less excess risk of cancer, potentially guiding clinical decision-making upon choosing a treatment.

2 LITERATURE REVIEW

2.1 KNOWLEDGE GAPS AND NOVELTY

***Table 1**. A selected overview of identified knowledge gaps and scientific novelty of the studies at the time each study was initiated.*

	What is already known?	Identified knowledge gaps	The scientific novelty at the time of study initiation
Study I	Menopausal hormone therapy (MHT) is still the only evidently effective prescription drug to alleviate the core symptoms of menopause. However, the total cancer risk associated with MHT use is incompletely understood.	1. What is the total risk of cancer associated with contemporary use of MHT? 2.What is the risk of the major cancer types? 3. Are there differences between various MHT treatment options?	This was the first sufficiently powered population-based cohort study evaluating the net effect of MHT use on cancer risk.
Study II	Ovarian cancer has a poor prognosis, owing to diagnostic delay and treatment resistance. Menopausal hormone therapy might increase ovarian cancer risk, yet many prior studies have been small with limited power. Evidence is sparse to determine whether the risk could differ by administration route of MHT treatment.	1. Does the risk of ovarian cancer vary between the various MHT treatment options prescribed in Sweden? 2. Does the risk differ by age at treatment initiation? 3. What is the risk among current and past users? 4. Does the risk change by administration route of MHT?	This was one of the largest population-based studies investigating the association between contemporary MHT use and ovarian cancer risk, taking advantage of the multiple MHT treatment options available in Sweden.
Study III	The gut microbiome may alter oestrogens' metabolism. Oral antibiotic treatment may lead to dysbiosis, possibly promoting inflammation and changing the microbiome's composition and even functions. Exposure to systemic oral antibiotics has been linked with an elevated colorectal cancer (CRC) risk, yet it is unclear if this posed association may vary within the colorectal continuum.	1. Does the risk of CRC differ within the colorectal continuum? 2. Does the risk vary between broad- and narrow-spectrum antibiotics? 3. What is the shape of the risk pattern? 4. What is the overall association between oral antibiotics with CRC risk?	This was the first dose-response meta-analysis to date of publication, addressing whether a potential dose-response relationship could depart from linearity. This was also the largest meta-analysis investigating whether the posed link could differ between broad- and narrow-spectrum antibiotics.
Study IV	Evidence suggests that MHT use could lower CRC risk, yet fewer studies have focused on the association with mortality. While the prognostic outlook could be even better among women who received MHT before their CRC diagnosis, current evidence is inconclusive.	1. What is the association of prediagnostic use of MHT with CRC-specific mortality and all-cause mortality risk? 2. Do these potential associations vary by the different MHT treatment options?	This was one of the largest population-based cohort studies, including virtually all Swedish MHT users diagnosed with CRC during the study period, estimating the influence of MHT use before CRC diagnosis on cause-specific and all-cause mortality.

2.2 BACKGROUND

Despite surgical advances and improved treatments options,(23-25) cancer remain the leading cause of death globally.(24, 26, 27) In 2018 alone, malignant neoplasms accounted for almost 10 million deaths, underscoring the high burden of the disease, highlighting the need for preventive measures.(26, 27) Among women, cancer of the breast and colorectal continuum are the most common cancer types globally.(23, 24, 26, 28) Whereas the risk factors are both hormonal and non-hormonal,(16, 29-31) emerging body of evidence suggests that the widespread use of common prescription drugs might be associated with carcinogenic risk, progression of solid tumours, and possibly even cancer survival.(9, 16, 31, 32)

2.2.1 Cancer - the leading cause of death

More than half of all deaths occurring globally (60%) are cancer-related,(24, 26, 27) in spite of advanced treatment options and implemented population-level or regional screening programs.(23, 24, 33) Paradoxically evidence suggests that around about 30-50% of all cancers could potentially be prevented, mainly owing to modifiable risk factors, and earlier detection due to implementation of screening programs.(26, 33) These facts not only highlight the pivotal role of preventive measures but also underscore the importance of identifying potential risk factors, as naturally, to prevent cancer, one must first identify possible risk factors contributing to carcinogenesis.(25, 34, 35)

2.2.2 Pharmacoepidemiology

Compared with other outcomes such as cardiovascular diseases, where the effect of drugs can be expected to be relatively immediate, cancer development requires a substantially longer latency period. Therefore, any meaningful investigation of a possible association of prescription drugs with cancer promotion, progression, or potentially preventive effect should be evaluated longitudinally over time. This requires i) long-term exposure, ii) more extended latency period over time, iii) large sample size, and iv) most importantly, the study should be conducted without exposing healthy individuals to any excess risk or potentially harmful side effects.(36) Unequivocally, addressing this type of research by conducting a randomised controlled trial (RCT) would be ethically unjustifiable. However, observational pharmacoepidemiological studies based on real-world data and settings allow the evaluation of various drugs and their effects in a prospective cohort with sufficient follow-up time. Besides, large-scaled nationwide and population-based cohort studies,(37-40) facilitate the

statistical power, enhancing the generalisability of the results,(36) without exposing healthy individuals to any excess risks.

Against a common misconception, some of the conducted trials might be underpowered, leading to imprecise estimates, which might be challenging to reproduce. Additionally, trials like observational studies are often limited to specific populations,(36, 38, 41) introducing some type of selection. However, real-world data gathered from valid registries on a population level may even reflect the current clinical praxis to a greater extent than trials. Well-designed registry-based observational studies might include a more pronounced variety of participants, enhancing the obtained results' generalisability.

2.3 CANCER EPIDEMIOLOGY

Breast cancer is a major health threat globally, accounting for one-fourth of all incident cancers.(23, 24) In 2018, over 28,000 Swedish women were diagnosed with cancer, breast cancer remaining the leading cause of cancer-associated deaths among middle-aged women, accounting for one-third of all female cancers.(33, 42) Of other female reproductive organ tract cancers, 10% are gynaecological cancers (±48% endometrial, 26% ovarian, and 16% cervical cancer). Ovarian cancer, although rare, is the most fatal of gynaecological cancers, particularly in high-income countries.(25, 35, 43, 44) The prognosis is poor, with 5-year relative survival of less than 50 % and an expected 10-year survival below 40 %, owing to late detection, lack of modifiable risk factors and increasing treatment resistance.(35, 43, 44)

Colorectal cancer is among the top three most common cancers globally, and it is the second most common cause of cancer-associated deaths among both sexes, summing up to 10% of all cancer-related deaths. Overall, gastrointestinal tract cancers account for almost a quarter of the cancer deaths worldwide,(45, 46) and the upper-gastrointestinal cancers, except for gastric cancer, are listed within the global top ten cancer deaths.(28) Whereas the overall incidence of colorectal cancer is seemingly declining, especially in high-income countries,(45, 46) an incidence increase has been noted across several continents, particularly among individuals younger than 40-50 years. Notably, whereas younger age is usually a favourable factor, some evidence indicates that these malignancies among the younger people may even have a poorer prognosis.(47-50)

2.3.1 General risk factors

Although environmental, behavioural, and genetic predisposition unarguably contributes to a large proportion of cancer cases, these factors alone should not explain the globally increasing burden of cancer.(26, 29) Whilst the aetiology naturally differs between various cancer types, and for some malignancies, the underlying mechanisms are incompletely understood; in general, the risk factors are hormonal and non-hormonal.(3, 25, 29, 34, 51)

Infection is one of the well-known risk factors for cancer and a major global health concern, contributing up to one-fourth of the diagnosed cancers annually, with somewhat lower impact in high-income countries.(30, 52) Whilst the evidence for bacterial infections is less clear, several viral infections are associated with an elevated cancer risk. A classic example of viral infection influencing cancer risk is *Helicobacter pylori* and gastric cancer, and certain specific strains of the *Human papillomavirus* and cervical cancer.(30, 52) Whereas no specific pathogens are firmly confirmed for breast cancer,(53, 54) some strains of *Escherichia coli* and *Fusobacterium nucleatum* are suggested promoting development and possibly even prognosis of colorectal cancer, potentially involving a worse prognosis.(55-57)

2.3.2 Ovarian cancer

Among a family history of ovarian cancer, gene mutations in Breast Cancer gene 1 and 2 (BRCA1/ BRCA2) are well-established risk factors for ovarian cancer.(58) Other risk factors associated with higher ovarian cancer risk are endometriosis, current tobacco use, infertility, menopausal hormones, increasing age, and age at menopause.(43, 59) In contrast, parity, breastfeeding, oral contraceptive use, and tubal ligation are linked with reduced ovarian cancer risk.(60) Notably, all of these factors apart from tubal ligation are linked to anovulation and thus connected with a lower cumulative number of ovulatory circles during lifetime.(59)

2.3.3 Colorectal cancer

Several well-confirmed risk factors are identified for colorectal cancer including, a family history of colorectal cancer, adenomas, and serrated polyps, increasing age, and untreated inflammatory bowel disease.(61, 62) Other risk factors include male sex, Westernized diet, and sedentary lifestyle, all associated with slight to modestly increased colorectal cancer risk. However, some evidence suggests

that up to 70% of colorectal cancers might be related to dietary factors,(62) which could further support the gut microbiome's role in the formation and progression of colorectal cancer.(55-57)

In contrast, colorectal cancer screening is linked with diminished colorectal cancer risk and possibly even mortality due to earlier detection of cancer and polyps.(61) Furthermore, prescription drugs that either include or alter oestrogens' metabolism are suggested to have a pivotal role in potentially reducing the risk of sex hormone-related cancers.(63)

2.4 THE ROLE OF OESTROGENS

Hormones, in general, contribute to the expected growth of various tissues among both women and men, and they may influence the behaviour and metabolism of cells. A common hallmark featured for all cancer progression includes uncontrolled cellular proliferation, and modified metabolism.(64)

Oestrogens either can directly induce cellular proliferation, or oestrogen metabolites may stimulate mutations. Common to both of these pathways is that they ultimately lead to an increased cell division, thus increasing the possibility of mutations to occur.(64) Therefore, oestrogens might stimulate cancer promotion and progression of solid tumours, possibly playing a pivotal role in the aetiology of particularly oestrogen sensitive cancers. Some of these cancer types are globally common, such as breast cancer, while other cancer types such as ovarian cancer are rare with a nonetheless poorer prognosis, highlighting the need for preventive measures and early detection. However, the effect of MHT is strikingly different in breast and endometrial tissue, varying across the different MHT types.(7, 9, 65, 66) Consequently, it is essential to cautiously evaluate the influence of various MHT treatment options on cancer risk.

2.4.1 Potential underlying mechanisms

The role of oestrogens in cancer development is undoubtedly complex.(4) Whilst oestrogens are considered to have carcinogenic properties, in contrast, they might be protective of particularly sex hormone-associated cancers.(13, 14, 46, 67-69) The effect of oestrogens is believed to be mediated by two different nuclear receptors, oestrogen receptor alpha (ERα) and beta (ERβ),(63) and especially ERβ associated pathways are likely involved in the underlying pathophysiology. Oestrogen receptor beta promotes pro-apoptotic signalling, particularly in the colon, inhibiting inflammatory signalling, and progression of colorectal cancer is associated with loss of ERβ expression. (70, 71)

In experimental mouse models, ERβ in normal colon epithelia has been associated with a reduced inflammation of the gut,(72) likely by promoting pro-apoptotic signalling and thereby reducing the formation of a tumour. Besides, exogenous oestrogens have been suggested to influence inflammatory markers, reducing crypt's proliferation,(72, 73) and changes in the gut microbiota's diversity among colorectal cancer-induced male mice.(63) Evidence indicates oestrogens modifying the microbiome, and as discussed above, oestrogens appear to influence the inflammation of the colon as well. Experiments are required to determine if these changes in the microbiome are for the better or not. In terms of colorectal cancer risk, oestrogens are suggested to be protective, but whether this is because of the gut microbiome changes is not yet determined. However, considering that this proposed association of oestrogens with the gut microbiome might be modifiable, it could contribute to lower colorectal cancer risk,(63) and potentially even better prognostic outlook among individuals exposed to oestrogens before the colorectal cancer diagnosis.

2.5 MENOPAUSAL HORMONE THERAPY

*Scientific illustration by **INA SCHUPPE KOISTINEN** ©, www.inasakvareller.se*

2.5.1 One of the most controversial drugs invented

Menopausal hormones were among the most frequently prescribed drugs in the United States in the 1990s, and MHT is still the most effective licensed pharmacological treatment against vasomotor symptoms, which constitute the cardinal menopausal symptoms.(74) However, MHT has been described as probably one of the most controversial drugs ever invented, owing to the controversy of observational studies contra randomised controlled trials.(75) It is hardly feasible to even mention menopausal hormones without at least shortly referring to the Women's Health Trial conducted in the United States shortly after the millennium – a landmark ending the popularity era of MHT.(6, 9) The Trial found a 24% increased breast cancer risk among participants receiving postmenopausal conjugated equine oestrogens combined with medroxyprogesterone acetate.(9) The initially shown association of combination therapy with increased risk of invasive breast cancer remained in post-trial evaluation,(76) and the association with breast cancer has been confirmed in previous and recent epidemiological studies.(7, 77)

In the context of menopausal hormones, observational studies are often associated with a healthy user bias, healthy continuer bias, and even a prescriber effect.(75) However, it is noteworthy how several essential factors, such as women's age at trial entry and type of MHT treatment, are all too often dismissed when generalising the trial results to other populations and settings. A challenge worthy of discussion is that the Women's Health Trial evaluated the effect of conjugated equine oestrogens and their combination with medroxyprogesterone acetate, whereas oestradiol is the most prescribed oestrogen formulation in Europe,(6, 76) inherently challenging direct comparisons.

2.5.2 Prescription patterns

Before the 1990s, Sweden was one of the top European consumers of menopausal hormones, with over 30% of users with a median duration of four years. Around the millennium, nearly 40 million daily-defined doses were prescribed annually.(78, 79) The number of prescriptions dropped after studies reporting an increased risk of deleterious health effects, including a higher risk of invasive breast cancer and cardiovascular events.(9, 80-85) By 2005, the daily-defined doses had halved down to 20 million. Per contra, no immediately evident decline in the breast or gynaecological cancer incidence was noted following the drop of MHT prescriptions in Sweden, as shown in **Figure 1**. However, caution should be taken due to changes in dose recommendations and formulations over

the past decades, which challenges comparisons and could even make them misrepresentative.(78, 79, 82, 86)

Nonetheless, valid concerns have addressed that many women with moderate to severe symptoms of menopause may be left untreated out of fear of the association with cancer.(6, 81, 87) Whereas under-treatment not only influences the individuals' health negatively, it may also provide market opportunities for less serious treatment options,(6) without proper follow-up or without being licensed by the Swedish Medical Products Agency.

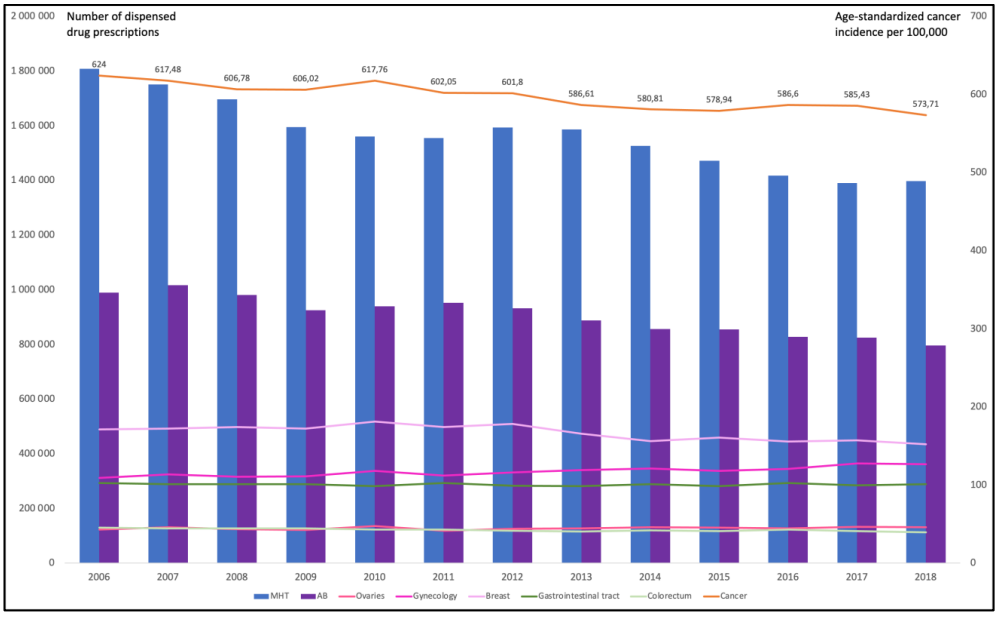

Figure 1. *Age-standardised cancer incidence among women aged 40 to 74 years, and the number of here investigated dispensed drugs, between 2006 and 2018 in Sweden. Data were retrieved from the National Board of Health and Welfare (https//: www.socialstyrelsen.se). Abbreviations: MHT: menopausal hormone therapy, AB: antibiotic treatment, Ovaries: ovarian cancer, Gynecology: all gynaecological cancers, Breast: breast cancer, Gastrointestinal tract: cancers of the gastrointestinal tract, Colorectum: colorectal cancer, Cancer: all malignant cancers.*

2.5.3 Treatment indications

Currently, the primary indication for menopausal hormone therapy is alleviating vasomotor symptoms, and menopausal hormones are not advocated for the prevention of chronic diseases.(4, 88, 89) The cardinal VMS symptoms include hot flushes and night sweats, affecting sleep, mood, and quality of life significantly. They are experienced by 50% up to 80% of women, possibly with some variation of the frequency across different ethnicities.(74)

Treatment initiation is recommended before 60 years of age or within ten years from menopausal onset.(90, 91) In general, treatment duration longer than five years is not advisable, yet some women may benefit from substantially longer treatment duration. Nonetheless, persistent vasomotor symptoms have been linked with an increased breast cancer risk,(92) warranting for a thorough individual risk-benefit assessment.(4, 6, 82, 87, 93, 94)

Whereas women with an intact uterus should receive only oestrogen combined progestin therapy (EP-MHT) to counteract unopposed oestrogens (E-MHT) associated risk of endometrial hyperplasia, the link between combination therapy and risk of breast cancer contra endometrial cancer is compelling.(7, 9, 95, 96) However, evidence is limited whether the association of menopausal hormones with the risk of cancer could vary between the different treatment options, and the net effect of MHT on cancer risk is understudied. Given as many as every third Swedish woman is expected to require MHT to alleviate the vasomotor symptoms,(6) it highlights the need to thoroughly investigate which treatment options could be safest, involving less excess risk of cancer.

2.5.4 Systemic versus local treatment

The indications for systemic versus local MHT treatment are different. Whilst systemic MHT is prescribed for the cardinal symptoms of menopause (*id est* vasomotor symptoms), local preparations such as vaginal creams are prescribed primarily for vulvovaginal atrophy. This thesis focuses on the contemporary use of systemic MHT, excluding the local treatments which should not be expected to have systemic effects.(97) This thesis included orally and transdermally administered systemic MHT preparations. Vaginal mucosa should be thicker compared to skin, and therefore, the vaginal preparations should not be absorbed into the bloodstream. Besides, any potentially absorbed amount from the local preparations should be minimal, not classified or described as systemic preparation.(97)

2.5.5 Oral versus transdermal treatment

Whereas oral and transdermal menopausal hormones effectively alleviate vasomotor symptoms, evidence indicates transdermal MHT involving a lower risk of cancer and other deleterious health effects, particularly thromboembolic events.(84, 85, 98) Transdermal MHT (*id est* gels, creams, and patches) requires a lower effective dose, as they are absorbed to the microcirculation, closely mimicking natural hormones and avoiding the first-passage in the liver. In comparison, oral MHT requires a higher dose following the significant loss of bioavailability due to the first-pass liver metabolism.(84, 98) While the harmful effect of oral MHT on venous thromboembolism risk appears to be associated peculiarly with medroxyprogesterone acetate, (84) less is known whether the association of transdermal MHT on the risk of cancer could vary between the different MHT types.

2.5.6 A unique feature of Swedish prescriptions

A distinguishing feature for prescriptions of MHT in Sweden is the availability of various treatment options and, in particular, the use of continuous and sequential combination therapy regimens. Combined with the valid high-quality Swedish health data registries, this offers a unique opportunity to investigate whether the association of contemporary MHT use and cancer risk may differ between the various MHT treatment options, based on longitudinal, large, and population-based cohort studies. Nonetheless, this unique Swedish feature also brings along several challenges. As mentioned earlier, comparisons to other studies conducted in countries with different MHT preparations and treatment options are complex. The doses and formulations have changed over time, and several of the MHT treatment options could be metabolised differently.(98)

2.5.7 Link with cancer

Even though menopausal hormones have been used over a half-century, its association with cancer remains inconclusive, requiring re-evaluation.(8, 9, 32, 99-102) Overall, it is becoming established that MHT use, in particular combination therapy, is linked with elevated breast cancer risk, whereas unopposed oestrogen-only therapy is associated with increased endometrial cancer risk.(8-10, 77, 96, 103, 104) The association with ovarian cancer is less clear.(105, 106) Per contra, a lower risk has been suggested for mainly male-dominant cancers such as cancers of the gastrointestinal tract, including oesophageal, biliary tract and pancreatic cancer.(12, 14, 15, 100, 107-110) Sparse evidence indicates a possible link between MHT use and central nervous system tumours.(111)

Whereas the mechanism of oestrogens for especially breast and endometrial cancer progression may be linked with increased cellular proliferation,(96) oestrogens do not seemingly pose direct effects on epithelial cells of the ovaries. However, ovulations are stimulated by hormones and followed by cellular proliferation, and a more significant number of cumulative lifetime ovulatory circles are linked to higher ovarian cancer risk, potentially supporting the role of cellular proliferation.(59)

2.6 ORAL ANTIBIOTIC TREATMENT AND RISK OF COLORECTAL CANCER

*Scientific illustration by **INA SCHUPPE KOISTINEN** ©, www.inasakvareller.se*

2.6.1 The global pandemic of antibiotic resistance

Antibiotics were developed back in the 1940s, and ever since, they have contributed to saving many lives. However, estimates suggest up to half of all prescribed antibiotics potentially being prescribed unnecessarily for non-indicated conditions.(112) Notably, this phenomenon contributes to the rise of new novel pathogen bacteria, fuelling the ongoing pandemic of global antibiotic resistance.(113, 114) Whereas Sweden has a somewhat more conservative prescription pattern of antibiotic treatment compared to several other European countries, approximately 20-50% of the world's population are annually exposed to antibiotics.(115-118)

2.6.2 Antibiotics, gut microbiome, and cancer

2.6.2.1 The gut microbiome

Typically, a healthy gut microbiome has several vital roles. These pivotal roles include reducing inflammation, producing essential vitamins, and functions related to oestrogen metabolism. An emerging body of evidence antibiotics changing the gut microbiome composition,(20-22) leading to a state known as dysbiosis and possibly altering important functions of the gut microbiome.(20, 119-121) Among some individuals, these effects of antibiotic treatment on the gut microbiome might be persistent or even permanent, allowing colonization with pathogens, and facilitating local inflammation and possibly even carcinogenetic events.(16, 29)

2.6.2.2 Antibiotics and oestrogens

Exposure to systemic oral antibiotic treatment has been posed as a risk factor for increased colorectal cancer risk,(18, 19, 122) and even more distal tumours such as breast cancer.(18, 31, 123) Whilst confounding by indication should be taken into consideration, it is biologically plausible that the impact of systemic antibiotic treatment on the gut microbiome could differ between broad- and narrow-spectrum antibiotics. In brief, broad-spectrum antibiotics could theoretically wipe out more of the normal gut microbiota, allowing for colonization with new pathogens, and potentially influencing the posed cancer risk to a greater extent as compared with narrow-spectrum antibiotics. Furthermore, the gut microbiome alterations shown among patients diagnosed with colorectal cancer may further imply the host-microbiome relationship's possible role. Additionally, antibiotics alter the metabolism of endogenous oestrogens, which could influence the risk of especially sex hormone-related cancers.(16, 29, 107) In fact, some data suggests that oestrogens and other chemopreventive

drugs such as non-steroidal anti-inflammatory drugs and aspirin could lower the colorectal cancer risk,(11, 105) which potentially indicates for inflammation's role in the formation of colorectal cancer.(16)

2.6.2.3 Posed cancer risk

The number of dispensed antibiotic prescriptions among Swedish women aged between 40 to 74 years has somewhat declined since 2012 (**Figure 1**), most likely owing to improved antibiotic stewardship. However, no apparent decline was seen in the incidence of gastrointestinal tract cancers.

There are significant knowledge gaps and a scarcity of solid evidence confirming the posed carcinogenic risk associated with exposure to oral antibiotic treatment and colorectal cancer risk. In specific, there is an unmet need for valid research to evaluate whether the risk of colorectal cancer might vary within the colorectal continuum or between the various antibiotic classes, in particular among broad- and narrow-spectrum antibiotics. Most importantly, none of the previously conducted meta-analyses has considered a departure from linearity,(18, 50, 124) although this is crucial to better understanding the shape of the potential risk pattern. A simple binary categorization to low versus high dose exposure to antibiotics could easily lead to loss of essential data, highlighting the value of understanding the risk pattern's shape.

3 RESEARCH AIMS

3.1 THE OVERALL AIM OF THE THESIS

This thesis aims to evaluate the association of two common prescription drugs on the risk of cancer. Specifically, this doctoral thesis aims to investigate the association between contemporary use of systemic MHT and the risk of cancer, its influence on colorectal cancer mortality, and clarify the posed association of oral antibiotic treatment with colorectal cancer risk.

Our primary hypothesis is that pharmacological treatment with prescription drugs, which either include oestrogens or may alter oestrogens' metabolism, might reduce the risk of sex hormone-associated cancers. For these cancer types, the prognostic outlook could potentially be even better among drug users than individuals who did not receive the drug.

3.2 SPECIFIC AIMS OF THE INCLUDED STUDIES

- To evaluate the total cancer risk among contemporary MHT users, compared to the Swedish background population.
- To assess the association between contemporary MHT use and ovarian cancer risk, evaluating which MHT treatment options may include less excess risk of cancer.
- To investigate the association between prediagnostic MHT use and risk of cause-specific and all-cause mortality among women diagnosed with colorectal cancer.
- To clarify the link of oral antibiotic treatment with risk of colorectal cancer through a systematic review and dose-response meta-analysis.

4 MATERIALS AND METHODS

4.1 OVERVIEW

An overview of the methods applied in studies I-IV is presented in **Table 2**.

***Table 2**. A selected overview of the methods in studies I-IV.*

	Study I	Study II	Study III	Study IV
Study design	Population-based cohort study	Population-based matched cohort study	Systematic literature review and dose-response meta-analysis	Population-based cohort study
Data sources	The Swedish Prescribed Drug Registry, Cancer Registry, Causes of Death Registry, and Registry of the Total Population	The Swedish Prescribed Drug Registry, Patient Registry, Cancer Registry, and Causes of Death Registry	PubMed, Web of Science, Embase, and The Cochrane Library	The Swedish Prescribed Drug Registry, Patient Registry, Cancer Registry, and Causes of Death Registry
Study Period	2005-2012	2005-2012	From inception up to February 2020	2006-2014
Inclusion	Women, at least 40 years or older at first recorded menopausal hormone therapy (MHT) prescription, who had received at least one prescription of systemic MHT during the study period		Individuals who ever used antibiotics	Women diagnosed with colorectal cancer who had received at least one dispensed MHT prescription before diagnosis
Primary outcome	The net effect on cancer	Ovarian cancer	Colorectal cancer	Colorectal cancer-specific and all-cause mortality
Main statistical analysis	Standardised incidence ratios	Multivariable conditional logistic regression	Random-effects meta-analysis	Multivariable Cox regression
Covariates	Age, sex, and calendar period	Hysterectomy, ever-parous, thrombotic events, year of birth, smoking-related diseases, alcohol-related diseases, obesity, diabetes mellitus, and osteoporosis	As reported in the original publications	Hysterectomy, ever-parous, thrombotic events, age at diagnosis, smoking-related diseases, alcohol-related diseases, obesity, diabetes mellitus, osteoporosis, cancer site, and stage of cancer

4.2 REGISTRY-BASED DATA SOURCES

An overview of the national registries used in the thesis included studies is presented in **Figure 2**. Data was ordered and received from the Swedish National Board of Health and Welfare, following a beforehand approved ethical application and a detailed *a priori* established study protocols for each of the studies included in this thesis.

Studies I, II, and IV were based on the high-quality Swedish registries with nationwide coverage of healthcare and other administrative data. The unique Swedish personal identity number ensured a valid and unambiguous linkage of data between the various registries. This personal identification number has been allocated to all Swedish residents since 1947. The Swedish National Tax Board, operating under the Swedish National Board of Health and Welfare's supervision, manages the identification number.(125-127) The Swedish Registry of Total population was established in 1749, yet incorporated into the Swedish Tax Office in 1962. However, the Population Registry is maintained by Statistics Sweden.(127, 128)

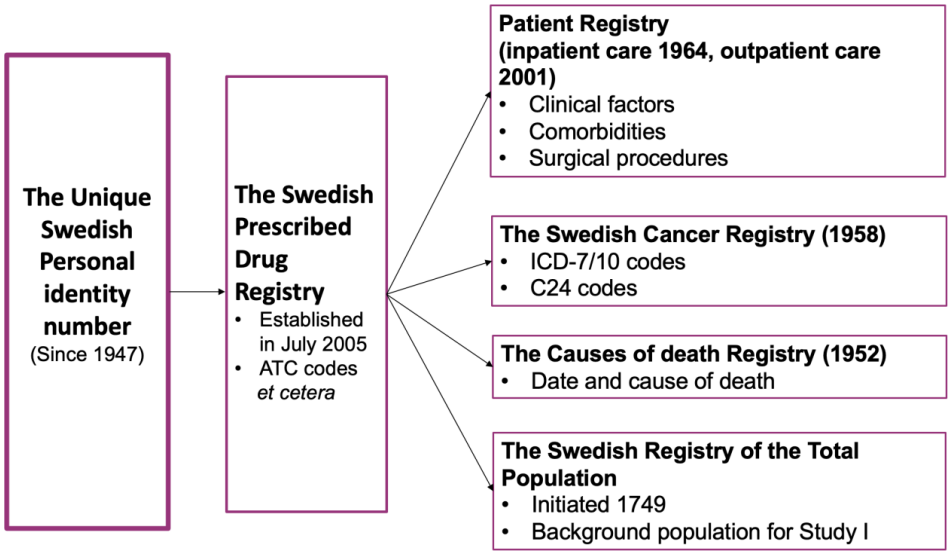

Figure 2*. An overview of the Swedish registries used in the studies included in this thesis. Abbreviations: ATC: Anatomical Therapeutic Chemical code, ICD-code: International Classification of Diseases code.*

4.2.1 Database for the registry-studies

To investigate the association of various MHT treatment options with the cancer risk and colorectal cancer mortality, we gathered a large cohort including virtually all Swedish women who had received at least one dispensed prescription of systemic MHT between July 2005 and December 2014 as identified from the Swedish Prescribed Drug Registry. This cohort of women was singled out from a database including over 7.5 million unique Swedish residents, covering more than 85% of the Swedish population, making it one of the largest cohorts globally to investigate prescription drugs' association with cancer-related outcomes.

The initial database included all unique residents who had received at least one dispensed prescription of *a priori* selected drugs (*id est* MHT, proton pump inhibitors, *Helicobacter pylori* eradication treatment, aspirin, and other non-steroidal anti-inflammatory drugs, statins, or bisphosphonates), as ascertained from the Swedish Prescribed Drug Registry.

The database had very high coverage of women at peri- and postmenopausal age. Over 90% of women aged 40-64 years and over 95% of women who were 65 years or older were included in the cohort.(7) **Figure 3** presents the initial cohort selection for Studies I, II, and IV. We only included women 40 years or older at the first recorded prescription date, excluding premenopausal women or individuals receiving menopausal hormones for other indications. Notably, the Swedish Prescribed Drug Registry was established first in July 2005. Thus, our data for prescription drugs started from July 2005.(125) Furthermore, considering previous cancer or cancer treatment could have influenced the results, all women with previous malignancy, apart from non-melanoma skin cancer, were excluded before cohort enrolment (Study I) or before the matching (Studies II and IV).

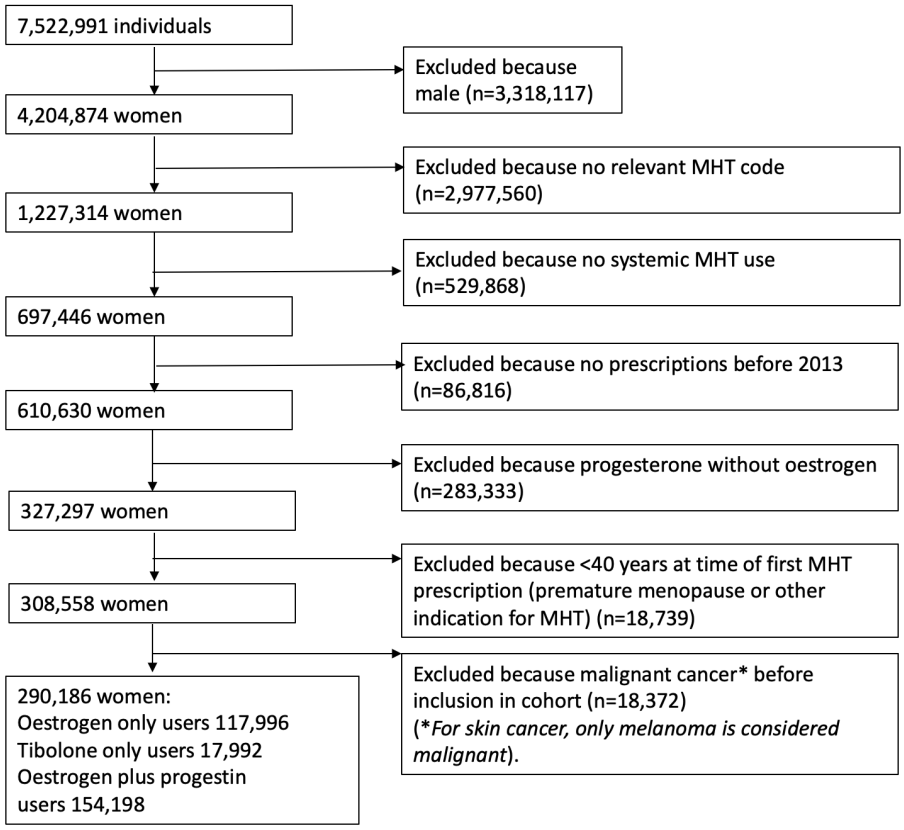

Figure 3*. The initial cohort selection of women exposed to menopausal hormone therapy (MHT), as identified from the Swedish Prescribed Drug Registry (Studies I, II, and IV).*

4.2.2 The Swedish Prescribed Drug Registry

The Swedish Prescribed Drug Registry was established in July 2005, and it is held at the Centre for Epidemiology at the National Board of Health and Welfare, operating under legislation issued by the Swedish government (SFS 2005:363).(125) The National Corporation of Swedish Pharmacies is responsible for the data collection, and information on dispensed prescriptions is reported monthly to the Centre for Epidemiology.(125)

The Prescribed Drug Registry covers virtually all outpatient care prescribed and dispensed drugs for all Swedish residents. Patient data are missing for less than 0.3% of all items. Prescribed drugs are entered into the Registry using Anatomical Therapeutic Chemical (ATC) classification codes (including substance, formulation, brand name, and package *et cetera*), and the Drug Registry includes data such as age, sex, number of prescribed prescriptions, date of prescribing and dispensing, dispensed amount and dose.(125) Whilst data on inpatient care used drugs is not included in the registry, menopausal hormone therapy is less likely prescribed in inpatient care settings. Thus, the coverage of the dispensed MHT prescriptions in the thesis included studies should be high. Noticeably, injectable, or over-the-counter sold MHT are not available in Sweden. **Table 3** presents the MHT treatment options considered in this thesis.

Type of therapy	Formulations/derivatives	Subgroup	ATC code
Oestrogen-only	Oestradiol (E2)	1	G03CA03
	Ethinylestradiol	1	G03CA01
	Oestriol (E3)	2	G03CA04
	Conjugated estrogens	3	G03CA57
	Other mixed	4	G03F
Tibolone only	Tibolone		G03CX01
Oestrogen + progestin	Continuous combinations (CC)		G03FA
	Sequential preparations (SP)		G03FB
Progesterone derivatives (Including natural progesterones)	Medroxyprogesterone acetate		G03DA02
	Medroxyprogesterone combined oestrogen	CC	G03FA12
	Medroxyprogesterone combined oestrogen	SP	G03FB06
	Progesterone		G03DA04
	Dydrogesterone		G03DB01
Testosterone derivatives	Dienogest		G03DB08
	Norethisterone		G03DC02
	Lynestrenol		G03DC03
	Norethisterone combined oestrogen	CC	G03FA01
	Dienogest combined oestrogen	CC	G03FA15
	Noretisterone combined oestrogen	SP	G03FB05
	Levonorgestrel combined oestrogen	SP	G03FB09
Other progesterones	Drospirenone combined oestrogen	CC	G03FA17

***Table 3**. Menopausal hormone therapy treatment options considered in this thesis. The subgroup presents the classification of oestrogen formulations. Abbreviations: ATC code: Anatomical Therapeutic Chemical Classification code, CC: continuous/ daily administration, SP: sequential administration.*

4.2.3 The Swedish Cancer Registry

The nationwide Swedish Cancer Registry was founded in 1958. Clinicians and pathologists are obligated by the Swedish law to report all new cancer cases to the Registry, and data is entered using International Classification of Disease (ICD) codes. Besides the date of diagnosis, information of the tumour type, site, and histology is recorded.(116, 125) The Registry has high completeness, with some variation across cancer types. Yet, preceding validation studies indicate a very high overall completeness (over 96%) and concordance.(116)

4.2.4 The National Causes of Death Registry

The Swedish Causes of Death Registry was established in 1952. Since 1984 and onwards, it has been held at the National Board of Health and Welfare. The Registry is 100% complete for deaths occurring in Sweden and records the date of death, cause of death, underlying causes of death, *et cetera* using ICD codes.(125, 129)

4.3 ELECTRONIC DATABASES

4.3.1 Published literature

Study III was a systematic review and dose-response meta-analysis for which the data was obtained from PubMed, Web of Science, Embase, and the Cochrane Library.

4.4 STUDIES ON MENOPAUSAL HORMONE THERAPY

4.4.1 Study I

4.4.1.1 Design

This was a nationwide and population-based cohort study with the primary aim of estimating the net effect of contemporary use of systemic MHT on the total cancer risk compared with the entire Swedish female general population. The secondary aim was to investigate the association between MHT use and risk of 16 different cancer types based on the incidence and mortality of cancer.(28, 130) The large-scaled and population-level design inherently increased the sample size, enabling the evaluation of various MHT treatment options and even more rare outcomes.

4.4.1.2 The cohort and follow-up

The cohort included virtually all Swedish MHT ever-users (N=290,186), who were 40 years old or older at first recorded prescription, and who had received at least one dispensed prescription of systemic MHT between 1 July 2005 and 31 December 2012, as ascertained from the Swedish Prescribed Drug Registry based on ATC codes. Non-users of menopausal hormones consisted of the entire Swedish female population at the same calendar period and age. The Swedish Cancer Registry was used to ascertain all cancer cases during the study period, classified by ICD 7^{th} and crosschecked with the ICD 10^{th} edition (Study I, eTable 1). Women with a history of malignancy, apart from non-melanoma skin cancer, were excluded before cohort enrolment.

4.4.1.3 Statistical methods

The relative risk of cancer was estimated by calculating standardised incidence ratios (SIRs) with 95% confidence intervals (CIs). The observed incidence of cancer among women exposed to menopausal hormones was divided by the expected cancer incidence among the entire Swedish female background population. We stratified the analyses by women's age at the start of the treatment (<60, 60-69, and ≥70 years), according to the recommended age for MHT initiation and cessation,(90, 91, 131) calendar period (2005-2006, 2007-2009, and 2010-2012), and MHT types. Women who received only E-MHT prescriptions were classified as oestrogen-only therapy users; those receiving one or more combination therapy prescriptions during the study period were considered EP-MHT users. Women were followed from the first prescription date until cancer development, death, or end of study (31 December 2012), whichever occurred first. Changes in the calendar periods and age groups were considered. The linear effect of age on cancer risk was

assessed using a score test for trend. Furthermore, to evaluate a possible influence of MHT type on the risk of cancer, we performed a regression with an interaction term, and statistical significance was tested with a Wald-test (alpha level set to 0.05). The first calendar period (2005-2006) was excluded from the sensitivity analyses. For analyses on the duration of use, treatment duration was categorized as having received menopausal hormones for <1, 1-2, 3-4, and ≥5 years based on daily-defined doses.

4.4.2 Study II

4.4.2.1 Design

This was a prospective population-level matched-cohort study investigating the association between contemporary use of MHT and the risk of ovarian cancer (ICD-7 codes 175.01, 9 crosschecked with ICD-10 codes C56, C57. 0-4, 7, 9) compared with women who did not receive MHT during the study period (2005 – 2012).

4.4.2.2 The cohort and follow-up

The Swedish Prescribed Drug Registry was used to identify all Swedish MHT users who were at least 40 years old at the first recorded prescription and had received at least one dispensed prescription of systemic MHT (N=288,950), between 1 July 2005 and 31 December 2012, as ascertained from the Swedish Patient Registry. Ever-users of MHT were group-level matched (1:3) to 866,546 women not receiving MHT during the study period. Firstly, we stratified women by three binary variables which may influence MHT prescription (*id est* hysterectomy, thrombotic events, and parity), and within each stratum, the exposed women were matched to the nearest neighbour (who did not receive MHT) on the year of birth, and main comorbidities (*id est* smoking- and alcohol-related diseases, diabetes, and obesity) based on discharge diagnoses (Patient Registry).(7) Women with a record of oophorectomy or tubal ligation were excluded to avoid misclassification, as identified from the Swedish Patient Registry.

4.4.2.3 Statistical analyses

Conditional multivariable logistic regression models providing odds ratios (ORs) with 95% confidence intervals were used to estimate the relative risk of ovarian cancer.(132) The estimates were adjusted for all the eight matching variables and osteoporosis (*id est* confounding by indication). Because the group-level matching aims to balance out the effect of follow-up time

between the groups, a conservative approach was chosen without assigning any substitute date for the non-exposed women to address the duration of MHT use.

All analyses compared ever-users of systemic MHT with women who did not receive menopausal hormones during the study period, and the analyses were stratified by age at treatment initiation (<60 years, 60-69, and ≥70 years). Exposure to MHT was classified based on ATC codes, and the various MHT treatment options were sub-grouped by MHT types, oestrogen formulations, and EP-MHT regimens. Here, tibolone users were separated from E-MHT users to investigate any potential differences associated with ovarian cancer risk. Women who changed MHT treatment during the study period were excluded from the sub-group analyses. Moreover, MHT users were classified into ever, current, and past users. Women who had received at least one dispensed prescription during the last six months of the follow-up were classified as current MHT users. All other women were classified as past MHT users.

4.4.3 Study IV

4.4.3.1 Design

This was a nationwide population-based cohort study within a larger population-based matched cohort, which is described in extensive detail elsewhere.(7) From the source cohort, we included all Swedish MHT ever-users and the group-level matched non-users who developed colorectal cancer between 2006 and 2012, ensuring exposure data availability before diagnosis (since July 2005).

4.4.3.2 The cohort and follow-up

In total, this cohort study included 7814 women who had been diagnosed with incident colorectal cancer between 1 January 2006 and 31 December 2012, as ascertained from the Swedish Cancer Registry. Women diagnosed with colorectal cancer (ICD-10 codes C18-C20) who received at least one dispensed prescription of systemic MHT before their cancer diagnosis, between 1 July 2005 and 31 December 2012, were considered prediagnostic MHT ever-users (n=1529). Women diagnosed with colorectal cancer not receiving menopausal hormones during the study period (n=6285), were classified as MHT non-users. The exposure was ascertained from the over 99% complete Swedish Prescribed Drug Registry. Oestrogen only, tibolone, and combination therapy prescriptions were classified based on ATC codes. Women were followed from cancer diagnosis until colorectal cancer-specific death (ICD-10 codes C18-C20, data available until 31 December

2013), all-cause mortality (ICD-10 codes A00-Z99, data available until 31 December 2014) or end of the study period (data available until 31 December 2014), as ascertained from the 100% complete Swedish Causes of Death Registry. Women who had undergone colorectal cancer surgery were identified from the Patient Registry (surgery codes are provided in Study IV, Supplementary Table 1).

4.4.3.3 Statistical analyses

We used multivariable Cox regression models to calculate hazard ratios (HRs) with 95% CIs for cause-specific and all-cause mortality risk among women diagnosed with colorectal cancer. All analyses compared MHT ever-users to MHT non-users and were adjusted for factors that might influence MHT prescription (*id est* hysterectomy, thrombotic events, and parity), age at diagnosis, smoking- and alcohol-related diseases, obesity, diabetes, osteoporosis (*id est* confounding by indication), site (*id est* colon or rectum), and stage of cancer (0+I, II, III, and IV, based on the Swedish guidelines). The different MHT types and the site of cancer were used to further stratify the analyses. Furthermore, MHT users were classified into ever, current, and past users based on the last dispensed prescription's timing. Women who had received at least one dispensed prescription within six months before colorectal cancer diagnosis were considered current MHT users, and all other women were classified past MHT users.

4.5 STUDY ON ORAL ANTIBIOTIC TREATMENT

4.5.1 Study III

4.5.1.1 Design

This was a systematic review and dose-response meta-analysis investigating the posed association between oral antibiotic treatment and the risk of colorectal cancer. **Figure 4** presents an overview of the study design *"step by step"*. No additional restrictions, such as language or geographical area, were set for the search. Eligible studies had to provide original data comparing individuals who were ever exposed to antibiotics to individuals not receiving antibiotics during the study period, providing standardised risk estimates from a cohort or a case-control study or a randomised controlled trial. Cross-sectional studies, case reports, and non-peer-reviewed papers, animal- and *in-vitro* studies, as well as publications with overlapping data, were excluded. The quality assessment of the included publications was conducted applying New-Ottawa Scale (NOS) for case-control and cohort studies,(133) and we used an additional customised quality assessment tool based on the most important key area's reported in each study.(134)

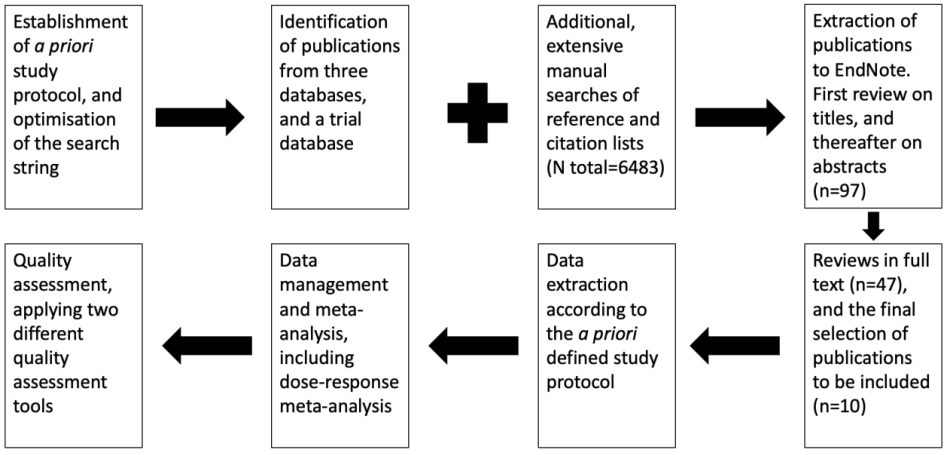

Figure 4*. An overview illustration of the various steps included in the study design (note: a detailed flowchart of the study selection is presented in the Results section).*

4.5.1.2 Statistical analyses

A random-effects meta-analysis was conducted pooling the most adjusted standardized risk estimates from the original publications, generating pooled effect sized (ES) with 95% CIs for each of the outcomes. These analyses compared individuals exposed to antibiotics with non-users during the study period. The non-users were defined as having received 0-1 prescriptions during the study period. We undertook multiple subgroup analyses by anatomical location, study design, indication, and the various antibiotics classes (as reported in the original publications).

Dose-response analyses were performed, exploring the potential dose-response relationship between the number of days exposed to antibiotics/number of prescriptions and colorectal cancer risk. Each prescription category's median was used to quantify the exposure as a continuous variable. Firstly, we fitted the dose-response model within each study and applied regression models to estimate each study pooled study-specific trends. A Wald test was utilized to evaluate potential deviation from linearity, and p <0.05 was considered suggestive of departure from linearity. Secondly, cubic splines were fitted, and the study-specific estimates were pooled in a multivariable random-effects model.(135) Statistical between studies heterogeneity was assessed using I^2 statistics classified as <50%, 50-75%, and >75%, indicating low, moderate, and high heterogeneity. Egger's test and funnel plots were employed to evaluate publication bias and small study effect.(136) As described in more detail in Study III, Supplementary Table 5, multiple sensitivity analyses were completed to evaluate the results' stability.

4.6 ETHICAL CONSIDERATIONS

The Swedish Ethical Review Authority (Etikprövnings myndigheten) granted the ethical approval for the studies I, II, and IV included in this thesis ("diarienummer" 2014/1291-31/4, principal investigator Nele Brusselaers). Informed consent was not warranted due to the studies' registry-based nature, without any biological material collection. Study III was a dose-response meta-analysis based on already published data.

The National Swedish Board of Health and Welfare performed the initially required registry linkages employing the unique Swedish personal identity number. The anonymized and deidentified data was received from the Board in encrypted form, and the code-key to identify any unique individuals is held only at the Board. All data was handled with great confidentiality, including highly restricted permission and accessibility only from the Centre for Translational Microbiome Research (CTMR) at the Department of Microbiology, Tumour and Cell Biology at Karolinska Institutet. All data is stored within the protected domain of Karolinska Institute's servers. Furthermore, no individual-level data is available for the cohort, and the results are presented at an aggregate level.

We anticipate no direct risks related to those included in the study, given the included studies' registry-based nature. The only potential threat to consider could be a violation of privacy; however, the risk of privacy violation is considered very mild given all data was received in anonymized and deidentified form, and all results were presented in an aggregated manner. Additionally, the research group has extensive experience in conducting large-scale registry-based research, and the group has performed multiple nationwide population-based registry studies.

5 RESULTS

In this thesis, all registry-based studies' results were reported according to the "Strengthening The Reporting of Observational Studies in Epidemiology" (STROBE) statement for cohort studies.(39) The systematic review and dose-response meta-analysis was reported based on Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines.(137)

5.1 STUDIES ON MENOPAUSAL HORMONE THERAPY

5.1.1 Study I

Most women (60%) exposed to systemic menopausal hormones were 60 years or younger at treatment initiation. Oestrogen-only therapy users were older at first recorded prescription compared to combination therapy users (**Table 4**). Overall, oestrogen-only therapy was less commonly prescribed (47.0%) than EP-MHT treatment (53.0%). The largest proportion of all exposed women (71.9%) received their first prescription in 2005 – 2006.

***Table 4**. Selected characteristics of the included women who ever used menopausal hormone therapy (MHT) between July 2005 and December 2012.*

Characteristics	Ever-users of MHT	Ever-users of oestrogen MHT	Ever-users of oestrogen plus progestin MHT
	Numbers (%)	Numbers (%)	Numbers (%)
Total	290,186 (100.0)	135,988 (46.9)	154,198 (53.1)
Age at first prescription			
40−49 years	46,299 (16.0)	14,196 (10.4)	32,103 (20.8)
50−59 years	127,773 (44.0)	43,385 (31.9)	84,388 (54.7)
60−69 years	59,592 (20.5)	28,887 (21.2)	30,705 (19.9)
≥70 years	56,522 (19.5)	49,520 (36.4)	7002 (4.5)
Year of first prescription			
2005−2006	208,555 (71.9)	100,090 (73.6)	108,465 (70.3)
2007−2009	46,736 (16.1)	20,553 (15.1)	26,183 (17.0)
2010−2012	34,895 (12.0)	15,345 (11.3)	19,550 (12.7)

Contemporary use of menopausal hormones was associated with slightly elevated cancer risk (SIR=1.09, 95% CI 1.07-1.11) (**Figure 5**). The overall association appeared to be stronger among combination therapy users (SIR=1.14, 95% CI 1.12-1.17) than oestrogen-only therapy users (SIR=1.04, 95% CI 1.01-1.06), yet the association was inconsistent for all cancer types. As shown in Study I, Table 2, the here noted increased cancer risk was linked to particularly female reproductive organ cancers (*id est* breast, ovaries, and endometrium) (SIR=1.31, 95% CI 1.28-

1.34), whilst the overall association was almost counterbalanced by the reduced risk of all cancers of the gastrointestinal tract (SIR=0.90, 95% CI 0.86-0.94). Among the group of gastrointestinal cancers, no apparent evidence was found for potential differences by choice of MHT treatment.

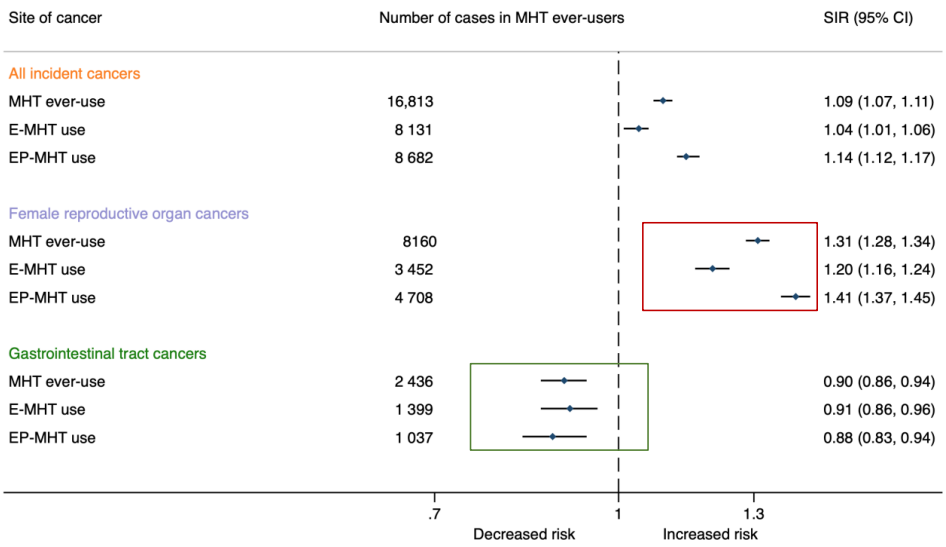

Figure 5*. The net effect of menopausal hormone therapy (MHT) on cancer risk, stratified by major cancer types and different MHT treatment options. The relative risk of cancer among MHT users, between 2005 and 2012, was compared to that of the female background population of the same age and calendar period. Abbreviations: SIR: standardized incidence ratio, 95% CI: confidence interval.*

Furthermore, the overall associations remained similar after excluding the first calendar period, and no apparent effect by the duration of MHT use was shown (Study I, Supplementary eTable 3). Of the various oestrogen formulations, a marginal association was shown for oestradiol (which accounted for 99% of the oestrogenic component in the combination therapy), whereas no association was found for oestriol (**Figure 6**). Per contra, tibolone use indicated for 14% increased cancer risk (SIR=1.14, 95% CI 1.08-1.21). Nonetheless, substantial differences were found particularly between the various EP-MHT treatment options, as shown in Study I, Table 3. In specific, continuously administered combination therapy regimes were associated with overall increased cancer risk. The highest risk was shown for the continuous testosterone-derived regimens (SIR=1.24, 95% CI 1.20-1.28), which was the most frequently prescribed EP-MHT regimen among the cohort (**Figure 6**). We found no overall association for sequentially

administered EP-MHT, neither progesterone-derived (SIR=1.09, 95% CI 0.91-1.29) nor testosterone-derived regimen (SIR=1.05, 95% CI 0.97-1.13).

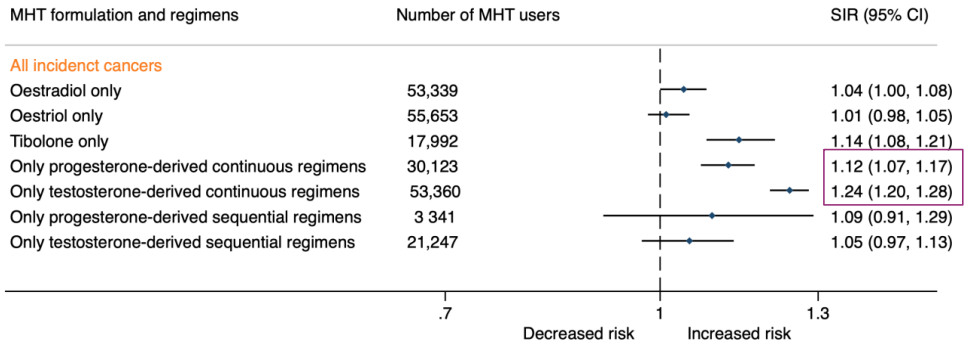

Figure 6*. The net effect of the different menopausal hormone therapy (MHT) formulations and regimens on cancer risk. The relative risk among women exposed to MHT between 2005 and 2012 was compared to that of the female background population of the same calendar period and age. Abbreviations: SIR: standardized incidence ratio, 95% CI: 95% confidence interval.*

5.1.2 Study II

This population-based matched cohort study included in total 1,155,496 women. Among them, 288,950 women had ever received MHT, and 866,546 group-level matched controls did not receive menopausal hormones during the study period between 1 July 2005 and 31 December 2012. As a result of the successful matching procedure, age and comorbidities were equally distributed among the groups (**Table 5**).

During the study period, 0.2% (n=572) of ever-users of MHT were diagnosed with primary ovarian cancer, and respectively, 0.4% (n=3588) of women who did not receive menopausal hormones. Of all ovarian cancers, 95% (n=3955) accounted for cancers of epithelial origin. Among women exposed to MHT, the vast majority (84.0%) received orally administered MHT. Out of the various MHT treatment options, oestrogen-only therapy (40.7%) was prescribed to fewer women compared to EP-MHT (53.1%), and 6.2% of the women received tibolone. Among EP-MHT users' testosterone-derived continuous regimes (34.6%) were the most prescribed.

Table 5*. The main descriptive characteristics of the study cohort.*

Characteristics	MHT ever-users N=288,950 Number (%)	MHT non-users N=866,546 Number (%)
All ovarian cancer	572 (0.2)	3,588 (0.4)
Epithelial ovarian cancer	542 (0.2)	3,413 (0.4)
Non-epithelial ovarian cancer	30 (0.0)	175 (0.0)
Age-groups, years		
<60 years	108,134 (37.4)	324,411 (37.4)
60-69 years	93,105 (32.2)	266,205 (30.7)
≥70 years	87,711 (30.4)	275,930 (31.8)
Menopausal hormone therapy		
All MHT	288,950 (100.0)	
Oestrogen only	117,506 (40.7)	-
Tibolone only	17,914 (6.2)	
Oestrogens combined progestins	153,530 (53.1)	-
Route of administration		
Oral	242,633 (84.0)	-
Cutaneous/transdermal	35,683 (12.4)	-
Oestrogen only users		
Oestradiol only	53,114 (45.2)	-
Oestriol only	55,424 (47.2)	-
Conjugated oestrogens only	1,159 (1.0)	-
Tibolone only	17,914 (6.2)	-
Oestrogen + progestin		
Progesterone derived only	47,095 (30.7)	-
Testosterone derived only	85,296 (55.6)	-
Mode of progestin administration		
Continuous only	91,982 (59.9)	-
Sequential only	28,132 (18.3)	-
Progestin regimens		
Progesterone derived, continuous	29,976 (19.5)	-
Progesterone derived, sequential	3,324 (2.2)	-
Testosterone derived, continuous	53,142 (34.6)	-
Testosterone derived, sequential	21,148 (13.8)	-
Clinical factors		
Ever parous (in-hospital delivery)	117,350 (40.6)	351,832 (40.6)
Thrombotic events	40,165 (13.9)	120,450 (13.9)
Hysterectomy	51,594 (17.9)	154,507 (17.8)
Diabetes Mellitus	15,868 (5.5)	48,229 (5.6)
Obesity	5,125 (1.8)	15,470 (1.8)
Alcohol-related disorders	7,264 (2.5)	21,351 (2.5)
Smoking-related disorders	13,551 (4.7)	40,826 (4.7)
Osteoporosis	8,222 (2.9)	22,654 (2.6)

Women who underwent hysterectomy with concomitant oophorectomy or salpingectomy were excluded from the analysis. Individuals receiving mixed therapies were excluded from the sub-group analyses. Abbreviations: MHT: menopausal hormone therapy.

Current use of combination therapy was associated with a 38% increased ovarian cancer risk (OR=1.38, 95% CI 1.18-1.62), whereas we did not find an association among the past user of EP-MHT (OR=1.00, 95% CI 0.84-1.18) or tibolone (OR=0.80, 95% CI 0.52-1.23) (**Figure 7**). In contrast, a strongly reduced ovarian cancer risk was suggested for oestrogen-only therapy users (OR=0.25, 95% CI 0.22-0.29%), regardless of past or current use. Furthermore, orally administered EP-MHT use (OR=1.23, 95% CI 1.08-1.39) suggested a more elevated cancer risk than cutaneous EP-MHT treatment, for which no association was found (OR=1.13, 95% CI 0.79-1.61), based on a smaller sample. As shown in Study II, Table 3, the risk estimates for cancers of epithelial origin resembled those associations shown for the overall ovarian cancer risk.

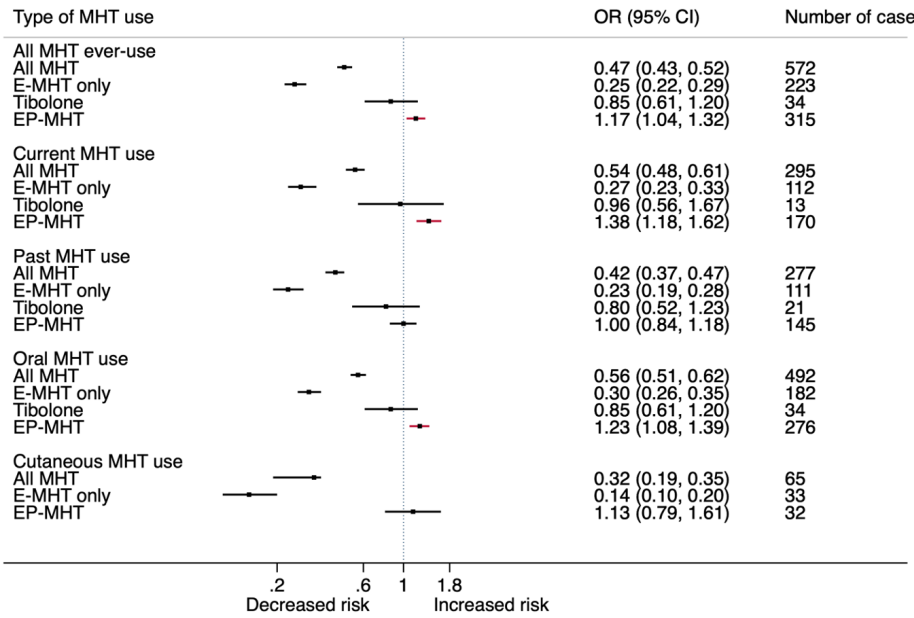

Figure 7. *The risk of primary ovarian cancer among Swedish users of systemic menopausal hormone therapy (MHT) between 2005 and 2012, compared with non-users, stratified by age at treatment initiation, the type of MHT, and current versus past use. Abbreviations: OR: odds ratio, 95% CI: 95% confidence interval.*

The association of different oestrogen formulations and combination therapy regimens with ovarian cancer risk is presented in Study II, Table 5. All the different oestrogen formulations were inversely associated with ovarian cancer risk, whilst we did not find an association for tibolone. Among EP-MHT regimens, the increased ovarian cancer risk was linked mainly to ever-use of continuous progestin derived regimens (OR=1.46, 95% CI 1.14-1.87) and current use of testosterone derived continuously administered regimens (OR=1.50, 95% CI 1.15-1.96).

5.1.3 Study IV

In total, this large nationwide cohort study included 7814 women diagnosed with colorectal cancer between 1 January 2006 and 31 December 2012, as identified from the population-level source cohort. Among these, 1529 women had dispensed at least one prediagnostic prescription of MHT prior to colorectal cancer diagnosis, and 6285 women, who were diagnosed with colorectal cancer, had not received MHT during the study period.

Overall, most of the women (77.2%) exposed to prediagnostic MHT had received their first prescription during 2005. As shown in **Table 6**, the source cohort's initial matching (on 1:3 ratio) remained rather stable, given age and comorbidities were similarly distributed among the groups. However, slightly larger proportion of menopausal hormone users were older than 60 years diagnosis (81.8%) compared to women not receiving menopausal hormones (79.6%). Hysterectomy was more common among women who did not receive menopausal hormones (30.8%) compared to prediagnostic MHT ever-users (25.7%). Notably, all the women diagnosed with colorectal cancer had undergone colorectal cancer surgery one year before the diagnosis and onwards. During the follow-up ranging from one year to eight years (median three years), a slightly higher proportion of women not receiving menopausal hormones died during the follow-up (42.0%) than hormone users (40.8%). Similarly, a somewhat lower proportion of MHT users died from colorectal cancer (27.3%) than MTH non-users (29.0%).

The findings of this nationwide and population-level cohort study indicate that past E-MHT use is associated with a 33% lower colorectal cancer-specific mortality risk (HR=0.67, 95% CI 0.44-0.99) and a 32% lower all-cause mortality risk (HR=0.68, 95% CI 0.59-0.93), compared with women diagnosed with colorectal cancer not receiving menopausal hormones during the study period (**Table 7**). However, differences were observed between the menopausal hormone treatment types and ages. Among current users, especially E-MHT was associated with elevated

all-cause mortality risk (HR=1.23, 95% CI 1.02-1.48) among women 70 years or older at colorectal cancer diagnosis. Per contra, the use of tibolone suggested a lower mortality risk (HR=0.44, 95% CI 0.18-0.99). (Study IV, Table 3). Notably, current use of combination therapy among women aged 60-69 years was linked with an elevated risk of colorectal cancer mortality (HR=1.61, 95% CI 1.06-2.44) (Study IV, Table 2). Also, current MHT use among women 70 years or older indicated for higher colorectal cancer-specific mortality risk (HR=1.31, 95% CI 1.05-1.65); however, the association attenuated in subgroup-analyses by the different MHT types (Study IV, Table 2). Furthermore, as shown in Study IV, Supplementary Table 3, we found no apparent differences were between the various oestrogen formulations.

Table 6*. The main descriptive characteristics of the study included women.*

	MHT ever-users Number of patients (%)	MHT non-users Number of patients (%)
Characteristics		
All MTH ever-users	1529 (100.0)	6285 (100.0)
*Pre-diagnostic current users**	829 (54.2)	
*Pre-diagnostic past users***	700 (45.8)	
Age at diagnosis		
<60 years	279 (18.3)	1282 (20.4)
60-69 years	529 (34.6)	1820 (29.0)
≥70 years	721 (47.2)	3183 (50.6)
Cancer site		
Colon	1061 (69.4)	4368 (69.5)
Rectum	468 (30.6)	1917 (30.5)
Site of cancer		
0+I	207 (13.4)	742 (11.9)
II	351 (23.0)	1441 (22.9)
III	359 (23.7)	1437 (22.8)
IV	250 (16.3)	1126 (17.9)
Unknown	362 (23.6)	1539 (24.5)
Year of diagnosis		
2006-2009	829 (54.2)	3544 (56.4)
2010-2012	700 (45.8)	2741 (43.6)
Type of MHT therapy		
Oestrogen-only	787 (51.5)	
Tibolone only	98 (6.4)	
Oestrogens combined progestins	644 (42.1)	
Clinical factors		
Ever parous (in-hospital delivery)	373 (24.4)	1620 (25.8)
Thrombotic events	370 (24.2)	1533 (24.4)
Hysterectomy	393 (25.7)	1953 (30.8)
Diabetes Mellitus	155 (10.1)	600 (9.5)
Obesity	30 (2.0)	106 (1.7)
Alcohol-related disorders	30 (2.0)	134 (2.1)
Smoking-related disorders	128 (8.4)	492 (7.8)
Osteoporosis	61 (4.0)	258 (4.1)

**Women who received at least one dispensed prescription of systemic menopausal hormone therapy (MHT) six months prior to colorectal cancer diagnosis were considered current users. **All other women were classified past MHT users.*

***Table 7**. The association between prediagnostic use of menopausal hormone therapy (MHT) and the risk of colorectal cancer-specific and all-cause mortality among the cohort included women diagnosed with colorectal cancer between 2006 and 2012 in Sweden.*

		Multivariable adjusted HR (95% CI) *	
		Colorectal cancer mortality	**All-cause mortality**
All MHT ever-users			
	All MHT	1.05 (0.93-1.18)	1.03 (0.93-1.14)
	Oestrogen-only	1.09 (0.92-1.29)	1.06 (0.92-1.21)
	Tibolone only	0.82 (0.53-1.28)	0.70 (0.46-1.09)
	Oestrogen + progestin	1.06 (0.89-1.25)	1.06 (0.91-1.22)
Current users			
	All MHT	1.15 (0.93-1.43)	**1.16 (1.01-1.32)**
	Oestrogen-only	1.15 (0.82-1.55)	**1.23 (1.04-1.46)**
	Tibolone only	0.57 (0.24-1.38)	**0.44 (0.18-0.99)**
	Oestrogen + progestin	1.25 (0.98-1.59)	1.16 (0.94-1.43)
Past users			
	All MHT	0.93 (0.79-1.09)	0.88 (0.79-0.99)
	Oestrogen-only	**0.67 (0.44-0.99)**	**0.68 (0.59-0.93)**
	Tibolone only	0.98 (0.58-1.63)	0.94 (0.56-1.56)
	Oestrogen + progestin	0.93 (0.73-1.16)	0.97 (0.80-1.18)
Anatomical location of the tumour			
	Colon	1.04 (0.90-1.19)	1.04 (0.90-1.19)
	Rectum	1.09 (0.84-1.40)	0.98 (0.79-1.21)

**Multivariable models were adjusted for hysterectomy, thrombotic events, ever-parous, age at diagnosis, smoking- and alcohol-related diseases, obesity, diabetes, osteoporosis, tumour location, and stage of cancer. The analyses compared the mortality risk among prediagnostic MHT ever-users to the risk among women not exposed to menopausal hormones during the study period. Abbreviations: HR: hazard ratio, 95% CI: 95% confidence interval.*

5.2 STUDY ON ORAL ANTIBIOTIC TREATMENT

5.2.1 Study III

This study was a systematic review and dose-response meta-analysis. A total of 6483 non-duplicate publications were identified, of which ten studies met the *a priori* defined eligibility criteria as shown in **Figure 8**. In total, this large study included over four million individuals and 73,550 individuals with colorectal cancer.

5.2.1.1 Included publications

These ten included publications addressed the link between oral antibiotics and colorectal cancer risk compared with individuals who had not received antibiotics.(19, 123, 138-145) The studies were published between 1998-2020 and came from highly developed countries: The United States of America, Netherlands, United Kingdom, Finland, New Zealand, and Taiwan. Apart from two studies, the publications were case-control studies. The characteristics of the meta-analysis included studies are presented in Study III, Supplementary Table 3.

All publications included only exposures to oral antibiotics. Data for cumulative antibiotic use was provided in six studies, whilst all apart from two studies reported antibiotic class-specific data. The number of antibiotic prescriptions was supplied in five publications, whilst the number of days exposed to antibiotics was reported in four of the publications, and only one publication utilised the cumulative dose expressed as tertials. Most of all included studies provided a lag-time of at minimum one year.

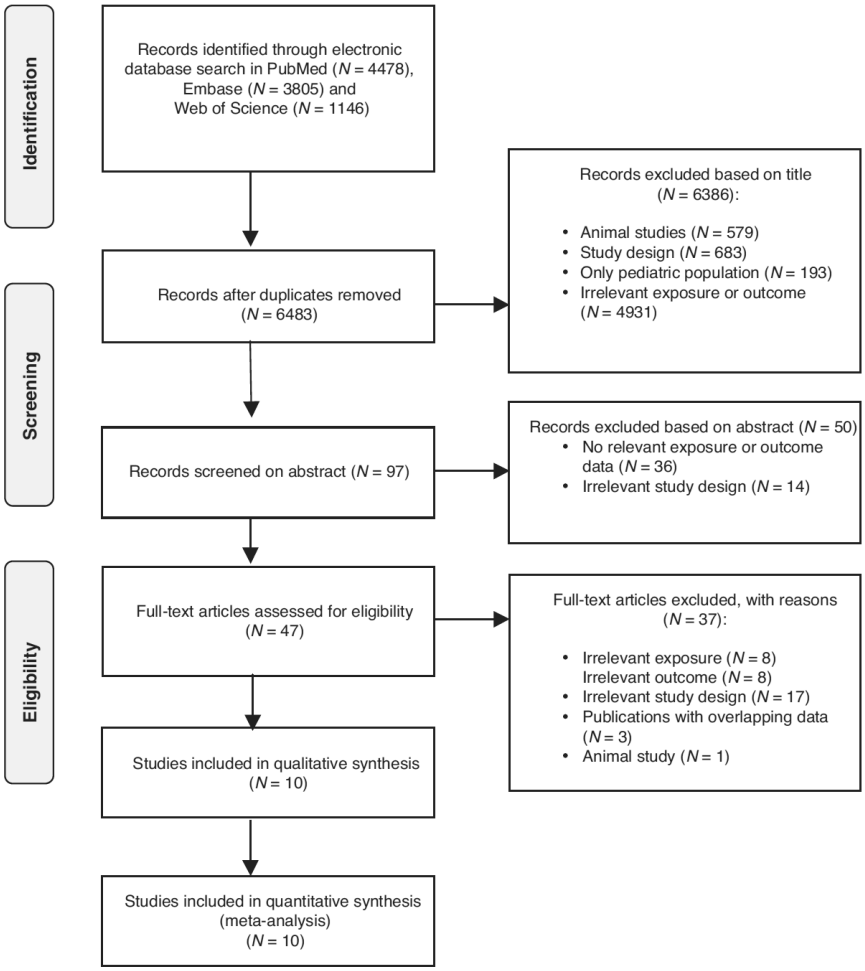

***Figure 8**. A detailed Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) flowchart of the literature search and the final selection of eligible studies.*

The overall risk of colorectal cancer following oral antibiotic use was 17% increased (ES=1.17, 95% CI 1.05-1.30, N=5) among individuals who were ever exposed to antibiotics. However, the association attenuated in stratified analyses for colon (ES= 1.06, 95% CI 0.89-1.26, N=4) and rectal cancer (ES=1.01, 95% CI 0.96-1.06, N=2) separately, based on fewer studies (**Figure 9**). Furthermore, as shown in Study III, Figure 3, the association with colorectal cancer varied across the various classes of antibiotics. A positive association was shown for penicillin, sulphonamides, quinolones, cephalosporins, nitroimidazole and metronidazole with effect sizes ranging between 1.16 to 1.33. A further subgroup meta-analysis restricted to only exposure to broad-spectrum antibiotics suggested a 70% (ES=1.70, 95% CI 1.26-2.30, N=3) increased risk among individuals ever exposed to these antibiotics, whereas we did not note any apparent link with narrow-spectrum antibiotics (ES=1.11, 95% CI 0.93-1.32, N=5), as shown in Study III/Supplementary Table 5.

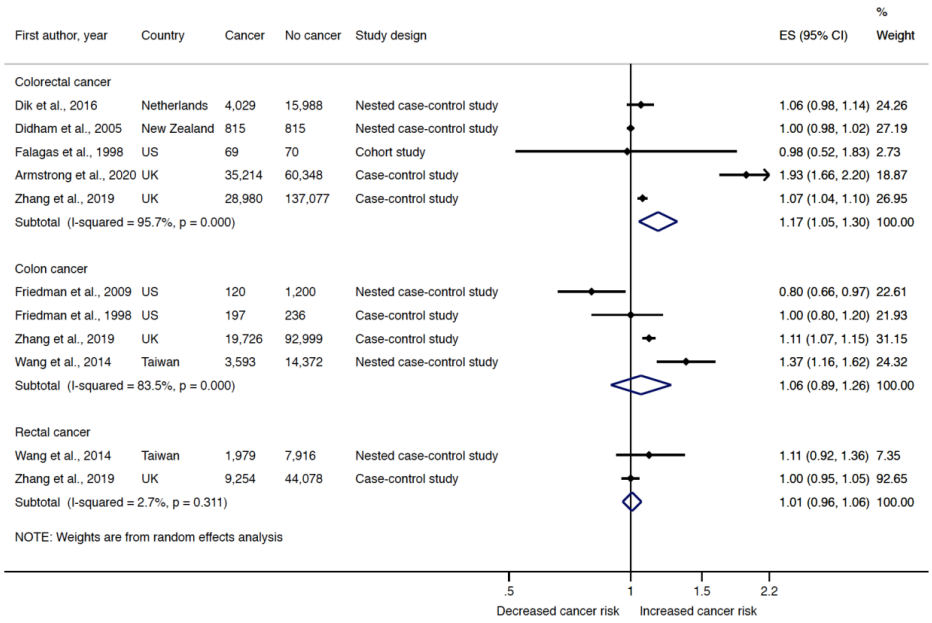

Figure 9. *Meta-analysis of the association of oral antibiotic use with colorectal cancer risk, pooling together the most adjusted risk estimates from the original publications in a random-effects model. Studies reporting antibiotic ever-use compared to non-use were included. Abbreviations: ES: effect size, 95% CI: 95% confidence interval.*

The association with colorectal cancer remained positive when restricting the analyses to only publications adjusting for non-steroidal anti-inflammatory drug use (ES=1.06, 95% CI 1.02-1.11, N=2), although seemingly somewhat attenuated (Study III, Supplementary Table 5). This study did not reveal any firm proof for a dose-response relationship, and the risk pattern's shape was similar within the colorectal continuum, as shown in **Figure 10**.

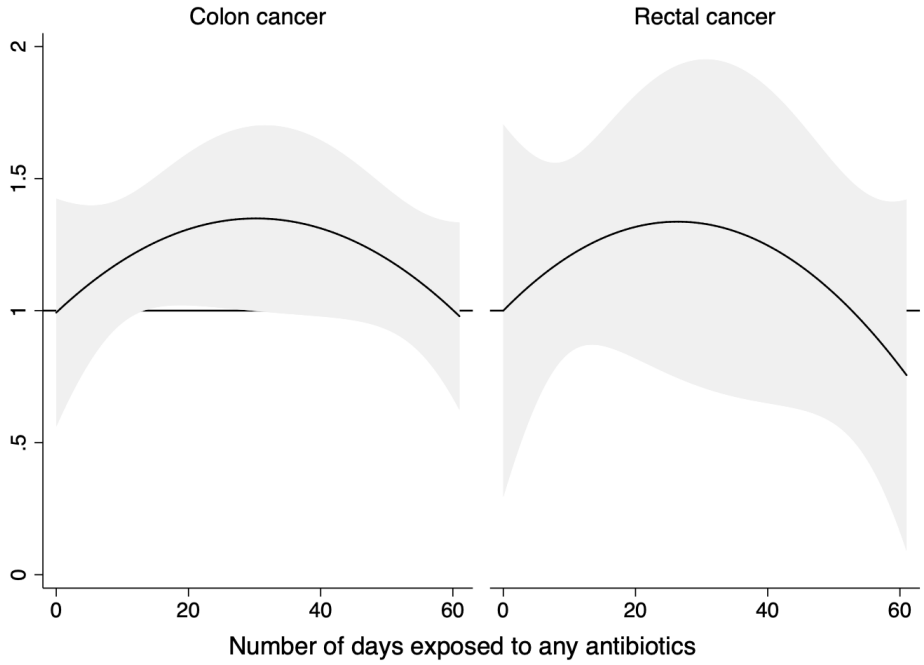

Figure 10*. A non-linear dose-response meta-analysis of the association between oral antibiotic use and risk of colorectal cancer, including studies reporting the number of days exposed to any antibiotics. The y-axis provides the relative risk (RR) of colorectal cancer, and the x-axis presents the number of days exposed to antibiotics.*

"Science does not aim at establishing immutable truths and eternal dogmas; its aim is to approach the truth by successive approximations, without claiming that at any stage final and complete accuracy has been achieved."

- *Bertrand Russell (1872 – 1970)*

6 METHODOLOGICAL CONSIDERATIONS

This thesis included three nationwide and population-level cohort studies based on the Swedish registries and one comprehensive systematic review and dose-response meta-analysis. Each of these studies had different methodological challenges and different opportunities to evaluate the studied association.

The methodological considerations for *Study III* are discussed in more detail in a separate section.

6.1 IT'S ALL IN THE METHODS

When one interprets the results of any scientific study, the concept of validity becomes of essential importance because the most important shortcomings arise from the methodological considerations. A poorly designed study with systematic and random errors is most likely to results in a wrong interpretation of the obtained results and imprecise estimates. Furthermore, there are also other caveats to consider, such as reverse causality, and to counteract the current reproducibility crisis that has shaken the scientific community; all studies should be reproducible.

In general, an inherent limitation of observational studies is the inability to prove causality. However, results from a valid study based on a scientifically sound methodology might provide evidence to support a causal relationship. Whereas one of the main purposes of scientific studies is to provide evidence, causality should be carefully assessed, given the complexity of real-world associations. At an absolute minimum, a causal inference would require a longitudinal study that is completely free from all systematic and random errors, showing a biologically plausible and strong association without any possible alternative hypothesis that could explain the observed results. To add yet another layer of complexity, a dose-response relationship is an essential dimension of the concept of causality, assuming naturally that the shown association is biologically plausible to begin with. It quickly becomes relatively clear that it is not feasible to draw causal inference from a single study, and the same type of bias may have influenced several studies showing the same association. Therefore, to draw a causal inference, it could be preferable that the results are consistent between studies applying different designs. However, some study designs are more vulnerable to certain types of biases.(146, 147)

6.2 STUDY DESIGN

Despite the complex nature of epidemiology, epidemiologic studies are conventionally classified in a binary manner to either descriptive or analytical studies. Notably, both study types include

various study designs aiming to answer different research questions, as illustrated in **Figure 11**. Because temporality cannot be ascertained in descriptive studies, these studies are suitable to describe distributions of a certain event or a disease at a given point of time among a defined population. In contrast, analytical studies may be used to investigate the strength and direction of associations in particular, and these studies become useful when exploring the aetiology of a disease. Pharmacoepidemiological studies are often of observational design, and they are frequently used to describe the effect of a given drug compared with individuals who did not receive the drug, applying real-world registry-based data. Furthermore, common measures obtained from longitudinal observational studies such as incidence and mortality may be used to evaluate, for example, the effectiveness of preventive measures or screening programs.(146, 147)

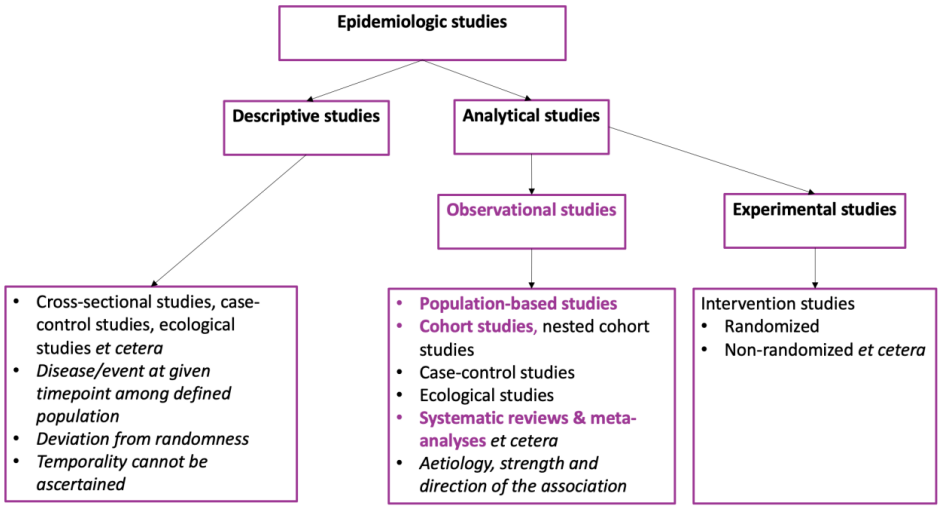

***Figure 11**. An overview of epidemiologic studies. Study designs highlighted in purple were included in this thesis.*

Occasionally interventional studies are not accounted as epidemiological studies, possibly indicating that epidemiologic studies are often considered being of more observational nature. However, it could be argued that experimental studies such as randomised controlled trials are also based on rigorous epidemiological methods. Whereas one could describe pharmacoepidemiological studies and trials as complementary, one of the largest differences between observational and experimental study designs is that it becomes overwhelmingly impossible to control for all the circumstances around the exposure in real-world settings. However, these real-world settings in a properly designed observational study may even reflect the clinical praxis to a greater extent compared with experimental designs.

6.2.1 Real-world data versus clinical trials

Whilst randomised controlled trials are often described as the golden standard, they are not suitable to answer all types of research questions. The benefit of pure randomisation lies in the fact that all potential confounding factors should be equally distributed among the study population by the effect of chance.(146) Given the complexity of real-world settings, identification of all potential confounders is challenging. In well-conducted trials, one might be more likely to control for confounders, which wouldn't have been considered otherwise – again owing to the effect of pure chance. That being said, several trials might be severely underpowered, inherently leading to imprecise estimates, which might even be challenging to replicate. Besides, randomised trials also involve some type of selection.(2, 36, 38, 41)

For example, MHT users are often described as somewhat healthier than women who do not receive MHT. This healthy user bias, and potential healthy continuer bias, may have distorted the found associations, particularly in observational studies.(40, 75) However, it is similarly questionable, who are the women participating in a randomised controlled trial? Couldn't they, as well, be healthier and more educated than women who opt-out of taking part in the trial? Therefore, it could be argued that rigorously designed population-level studies based on real-world data may reflect the true population more accurately as compared with trials, enhancing the generalisability of the results.

The registry-based studies included in this thesis aimed to explore the influence of menopausal hormones on cancer risk, and theoretically, the association could either lead to an increased or a decreased risk. Therefore, large clinical trials with sufficiently long follow-up time would not only

have been costly but, most importantly, unethical to conduct, given the potential risks could have over exceeded the possible health benefits.(2)

6.2.2 Cohort studies

The data for the thesis included registry-based studies were retrieved from the nationwide Swedish registries using the unique Swedish personal identity number, ensuring a valid data linkage. Given registration to these health data registries is mandatory,(148) the data is of high quality.

These studies' major strength was the population-level design reflecting real-world settings, inherently reducing the risk of selection bias, increasing the validity and generalisability of the results. Furthermore, the inclusion of virtually all Swedish women who ever received MHT treatment increased the statistical power to detect smaller yet meaningful associations, with sufficient power and follow-up time even for rare outcomes such as ovarian cancer (Study II) and for the different MHT treatment options. Overall, the high-quality Swedish registries have high coverage, completeness, and follow-up. Moreover, the risk of cancer and colorectal cancer mortality were evaluated longitudinally, ascertaining temporality. In contrast, many of the previous studies have been relatively small with power limitations, emphasizing the unmet need for valid and reliable large-scaled studies to clarify the role of MHT on cancer risk without exposing women to any potential additional risks.

6.3 VALIDITY OF STUDY

All risk estimates from scientific studies, no matter how rigorous the applied methods were, are, in fact, estimates of the true value among the studied population. A study's validity reflects how valid the study and the obtained results are. In general terms, the concept of validity is subdivided into internal and external validity. A study with good internal validity leads to higher external validity, enhancing the results' generalisability.(146)

Internal validity is to a large extent dependent on systematic errors and various caveats in the methodological considerations, such as reverse causality or confounding by indication. Furthermore, internal validity cannot be achieved without reasonable reliability, meaning that the results should be reproducible in the future, and therefore this concept is closely related to measurement and transparency.(146)

6.3.1 Internal validity

Internal validity attributes to how well a study was conducted, and this can be mainly influenced by systematic errors and the following shortcomings in the applied methods. Notably, these types of errors are not dependent on chance, and they could lead towards biased results through i) selection bias, ii) information bias, iii) and confounding.(146)

All the studies included in this thesis applied solid methods based on *a priori* defined study protocols. The high-quality registries and the inclusion of virtually all Swedish users of systemic MHT (Studies I, II, and IV) enhanced the validity of the results. The data is representative to date, as there have been no considerable changes in clinical praxis after the study period.

6.3.2 Selection bias

The risk of selection bias is a general concern in observational studies and can lead to biased associations. Whereas the risk of systematic errors, including selection bias, cannot be entirely eliminated by increasing the sample size,(146) a population-based approach counteracts the extent of a possible selection bias, as it inherently improves the representativeness of the population. To additionally alleviate concerns of a potential selection bias, we took advantage of the variety of MHT treatment options prescribed in Sweden. We sub-grouped our analyses by the different treatment options (Studies I, II, and IV) and current versus past use of MHT treatment.

However, although access to healthcare in Swedish settings should be equally distributed among the population, women using MHT might be healthier than women who were not prescribed MHT. Therefore, healthy user bias may be of concern, as a possible link between MHT use, higher socioeconomic status (including higher education and wealth), better health, and possibly even better survival has been described.(68, 149) On the other hand, women receiving MHT might experience more severe symptoms of menopause, warranting for treatment. Given women with more severe symptoms may have different levels of circulating hormones or alterations of levels during the menopausal transition, this could lead to a mixture of both endo- and exogenous hormones.(150)

Another dimension of the healthy user bias is a healthy continuer bias. In such cases, women using MHT treatment could be more prone to adhere to (any) treatment compared with women not receiving menopausal hormones. Likewise, clinicians may be more prone to prescribe MHT to women who are in general healthier, and MHT is contraindicated among women who have a high

risk of breast cancer, women with unclear vaginal bleeding, and women with arterial or liver disease.(91, 151, 152)

Altogether, these facts may contribute to the group of MHT users representing somewhat healthier women and limiting the generalisability of the results to healthier women without previous malignancy or contraindications. However, due to the successful group-level matching procedure in Studies II and IV, clinical factors and comorbidities were relatively equally distributed among MHT ever-users and MHT non-users.

6.3.2.1 Lead-time bias and over-diagnosing bias

Users of MHT treatment may utilize healthcare services to a greater extent as compared with MHT non-users. Consequently, this could lead to earlier cancer detection, selectively among users of menopausal hormones, potentially either over or underestimating the risk estimates.(78, 153) A possible over-diagnosing bias could be of concern, particularly for breast cancer, given mammography has been debated for the related false-positive findings.(33) This could have theoretically resulted in an overestimation of breast cancer in Study I. However, it would unlikely explain the different MHT types' shown differences, and in the previous Nurses' Health Study, the positive association with breast cancer remained significant after adjustment for mammography, although attenuated.(154)

On the other hand, cancer survival depends largely on the stage of cancer but also the availability of screening programs and treatment.(146, 147) In Study IV, screening efforts for colorectal cancer could have led to earlier detection and removal of polyps, thus diminishing colorectal cancer risk among this group. However, there were no existing population-level screening programs for colorectal cancer in Sweden for the time being. Nevertheless, in Study IV, all the women diagnosed with colorectal cancer, from one year before the diagnosis and onwards, had undergone colorectal cancer surgery, which could indicate curative intent.

6.3.3 Information bias

Information bias might arise when exposure, outcome, or a confounding variable is misclassified. Depending on whether these potential errors in measurement could lead to non-differential or differential misclassification, they could either dilute the risk estimates towards null or result in an over-or underestimation of the results.(146)

All the studies included in this thesis were based on meticulous study protocols, including detailed information on both the exposure and outcome. Study III was a systematic review and dose-response analysis, yet most of the included publications were registry-based studies reducing recall bias risk. In the thesis included registry-based studies (Studies I, II, and IV), the exposure was ascertained from the highly complete (>99% for outpatient care drugs) Swedish Prescribed Drug Registry.(125, 155) All data on exposure was extracted in the form of ATC classification codes, minimising the risk of misclassification of exposure per se. To further reduce the misclassification of treatment type, women who received one or more oestrogen combined progestin prescriptions were classified as EP-MHT users. As a result, users of unopposed oestrogen-only therapy were strictly differentiated from women who ever received progestins, forming more homogenous groups. Furthermore, menopausal hormone therapy is only available on prescription in Sweden, and therefore over-the-counter drug use is improbable to have led to misclassification of the exposure. This type of misclassification could only occur when and if women bought their MHT treatment abroad. Still, this scenario is less likely to contribute to any significant amount of neither prescriptions nor MHT users.

We cannot ignore the possibility of left censoring as most women (≈60% in Study I and II) were enrolled in 2005 because the Swedish Prescribed Drug Registry was first established in July 2005.(125) This could introduce a potential misclassification of exposure and followingly age at treatment initiation. However, possible exposure misclassification should occur at random between the groups, diluting the estimates towards the null. Nonetheless, an accurate duration of MHT use is important, particularly if assuming causal association, as long latency-period is expectable for cancer-related outcomes. Therefore, to explore the potential influence of left censoring, we performed sensitivity analyses excluding all women with the first prescription of MHT in 2005 (Study I), and the results remained similar, suggesting the robustness of our results. Another inherent study-specific limitation for Study I is that women exposed to MHT were part of the background population, which could dilute the risk estimates towards the null. This may be particularly problematic for those cancer sites for which no apparent association was shown, and therefore the results should be interpreted with caution. Nonetheless, the study provided vital information on the net effect of menopausal hormones on cancer risk. In Studies II and IV, we distinguished present MHT users from incident users, stratifying the analyses by current and past users. Furthermore, the group-level matching aimed to reduced statistical uncertainty and to neutralise the effect of follow-up, enhancing the groups' comparability.(7)

The Swedish Cancer Registry, which is generally over 96% complete, was used to ascertain cancer in Studies I, II and IV. Data is entered into the registry based on ICD-codes.(116) However, data for some cancer types, such as liver cancer, may be underreported in the Swedish Cancer Registry, owing mainly to diagnostic methods changes.(151) The Causes of Death Registry is 100% complete for deaths occurring in Sweden,(129) and any potential misclassification (*id est* cancer-related death) should occur at random, thus potentially diluting the estimates. Whilst, in general, determining the cause of death might be challenging, in Study IV, all women who died from colorectal cancer were diagnosed with colorectal cancer at the entry of the study. This might alleviate concerns related to colorectal cancer-specific death's accuracy, yet it may indicate tumour recurrence. Furthermore, the Swedish Patient Registry is nationwide complete since 1987 for in-patient care records, including surgery codes.(156) Therefore, some of the hysterectomies and oophorectomies may have been underreported, particularly among older women. This limitation could explain the observed strongly reduced risk of ovarian cancer risk among women receiving oestrogen-only therapy in Study II.

6.3.4 Confounding

Confounding is a general concern for all observational studies. A confounder is a factor correlating with the exposure, while it is simultaneously a self-reliant risk factor for the outcome. However, it cannot be an intermediate factor on the pathway from exposure to the outcome. Uncontrolled or inadequately controlled confounding may lead to a mixture of effects and residual confounding, ultimately leading to a biased association and wrong interpretation of the results.(146)

In the thesis included registry-based studies confounding was taken into consideration at three stages: i) design of the study, ii) collection of the data and iii) analysis of the data.(146) The population-based approach avoided selection of individuals, both exposed and non-exposed, given the high coverage of the Swedish Prescribed Drug Registry.(125) Whereas the inability to retrieve information on confounders for the entire Swedish female background population was an inherent limitation of the design in Study I, age is one of the major confounding factors for cancer. Additionally, standardisation by year of birth facilitates comparison with other studies and populations.(157, 158) Whereas stratification may come at the cost of external validity, stratification by age at treatment initiation was justified as it provided clinically valuable information, which can be used as an aid in clinical risk assessment. In studies II and IV, confounding was controlled in the study design by means of the group-level matching, and the

multivariable regression models were adjusted for several potential confounding variables and osteoporosis.

Nonetheless, some registry variables might be more prone to capture the most severe cases, and the possibility of residual confounding cannot be ruled out. Obesity is often underreported, and the variables for alcohol- and smoking-related diseases may have captured individuals with heavier drinking and smoking habits. Moreover, data were lacking on dietary oestrogen intake, which could lead to an increased cumulative oestrogen intake during a lifetime. Yet, correct measurement of dietary factors is challenging as the standardised questionnaires also have limitations, and it is unclear if dietary exposure to oestrogens could be sufficient to contribute to carcinogenesis or mortality. Furthermore, data on previous oral contraceptive use was unavailable for our cohort. However, in Sweden, the users of oral contraceptives are very young concerning the studied outcomes, and therefore less likely to have influenced our results.(159)

6.3.4.1 Confounding by indication

Confounding by indication arises when the exposed individuals differ from the non-exposed persons by the indication of the prescribed drug.(146) Whereas alleviation of the vasomotor symptoms is the primary indication for MHT, menopausal hormones are also prescribed for osteoporosis. Therefore, we adjusted the analyses for osteoporosis in Studies II and IV. However, confounding by indication could be a larger issue for exposure to antibiotic treatment (Study III), given individuals with a high cumulative intake of oral antibiotics could have an underlying chronic disease warranting for the treatment. However, this might be more problematic for other cancer types such as lung cancer, given these individuals would be more likely to receive high cumulative doses of antibiotic treatment for infections of the respiratory tract.

6.4 RANDOM ERROR AND PRECISION

Even if a study would be completely free of systematic errors, the study may not necessarily be free of random errors. These random errors occur due to random fluctuations in the sampling, causing variation in the data that cannot be explained. Random errors can be minimised by randomised sampling or as representative sampling as possible.

Precision could be defined as an absence of random error, providing an estimate of the uncertainty of the results. It depends both on the size of the study and prevalence of the exposure, and thus a large sample size inherently narrows the width of the confidence intervals, providing more precise estimates.(146)

To measure precision, statistical hypothesis testing providing *p*-values and calculation of confidence intervals is often applied. However, as observational studies are not randomised, the dichotomised statistical null-hypothesis testing may not be a preferable approach.

6.4.1 Type I error

Type I is a random error arising when a statistically significant association is shown, although, in reality, no such association exists in the study cohort. Thus, alpha error equals rejecting a true null hypothesis.(146) As an example, type I error could mean that we show an association between MHT use and cancer risk, although, in reality, no such association would exist.

6.4.2 Statistical significance testing

An alpha level of 0.05 provides a statistical significance level for committing an alpha error, meaning we accept the results occurring due to pure chance 5% of the time (160, 161). *P*-values provide information indicating the likelihood of finding the observed association or, more extreme, due to chance given the null hypothesis is true (*id est* there is no association).(146, 147) Occasionally, *p*-values are misleadingly interpreted as a simple probability. However, the *p*-value is only a threshold value for statistical significance testing, providing a cut-off level to support binary decision-making in terms of either keeping or rejecting the null hypothesis. Notably, *p*-values do not necessarily confirm the alternative hypothesis to be true, even if the null hypothesis can be rejected. In return, even if the results are not significant, this can only be interpreted as an absence of evidence to reject the null hypothesis, which could be explained by various factors such as sample size. Therefore, the lack of statistical significance does not confirm that there would not be an effect. On the contrary, the risk with this type of binary null-hypothesis testing is that it might

lead to a simplistic dichotomous interpretation of the data, which is questionable given the complexity of real-world associations.(160, 162, 163) It could be more beneficial to assess the found association in terms of theories, which are not based on chance, such as systematic errors.

6.4.3 Type II error

Type II error arises if no statistically significant association is detected, although there is a true association in the study cohort.(146) This equals retaining the null hypothesis when it is false, and it could occur due to random noise or small sample size.

The sample's statistical power is the capacity to appropriately distinguish differences, thus rejecting the null hypothesis when it is not true. The power is conventionally set to 80%. The likelihood of carrying out a type II error is referred to as β-error, and the power of the sample is calculated by the following formula:

$$Power = 1 - \beta\text{-}error.$$

The conventionally accepted level of β-error is up to 20%, meaning we accept that we don't find a true association in 20% of the cases.(146)

In this thesis included Studies I, II, and IV, the nationwide and population-based approach enhanced statistical power. Yet, rare cancer types had naturally smaller sample sizes, limiting the statistical power, particularly for subgroup analyses. Therefore, some true associations may have been missed due to power limitations. Study III was a systematic review and dose-response analysis summarising and mathematically synthesising previous knowledge, increasing the sample size and power. However, as for the dose-response analyses, it is expectable that the exposure is skewed, as fewer people are likely to receive high cumulative doses of antibiotics. Thus, the data for the dose-response relationship is likely more solid for the lower number of accumulated doses.

6.5 CONFIDENCE

Confidence could be defined as not finding an association when there is no association. Thus, confidence would be equal to stating that MHT treatment has no association with cancer risk, given there is no such association in the real world either.

Confidence is calculated by the formula:

$$Confidence = 1 - \alpha \ error.$$

P-values provide us with information on whether the confidence interval overlaps with zero or not, yet they are not open or indicative of the uncertainty of results.(160) Confidence intervals, however, present many different aspects. In a simplistic manner, it could be stated that if one performs the measurement 100 times, 95% of the time, the measurement will be within the confidence intervals. However, confidence intervals provide not only the estimated effect but also the degree of uncertainty and the direction and magnitude of the association. Yet, the role of *p*-values and confidence intervals is heavily dependent on the sample size, assuming an absence of selection bias, information bias, and confounding. Therefore, the results of a scientific study should be evaluated in the light of biological plausibility, previous knowledge and results, potential risk-benefit ratios, not to forget data quality *et cetera*.(163)

6.6 EXTERNAL VALIDITY

The study's external validity describes how well the obtained results can be generalisable to other populations and settings. Studies I, II, and IV included almost all Swedish users of menopausal hormones during the study period, facilitating the generalisability of the results to similar populations with comparable demographics, access to health care, related diagnostic criteria, applied treatment, and similar lifestyle habits.

However, as we excluded all women with a history of cancer at the beginning of the study or before the matching procedure, our findings might not be as generalisable to women with previous cancer or who have received cancer treatment. Whereas not all women diagnosed with colorectal cancer (in entire Sweden) were included in Study IV, the study included all MHT ever-users diagnosed with primary colorectal cancer during the study period.

Study III included publications across various continents. However, all studies were based on populations from highly developed countries, limiting the generalisability of the results to populations with similar demographics and comparable antibiotic prescription praxis and stewardship.

6.7 SYSTEMATIC REVIEWS AND META-ANALYSES

A meta-analysis is a mathematical way to quantify and produce new knowledge based on previous publications. As compared with other study designs, meta-analyses inherently increase the sample size, enhancing the statistical power, increasing the precision of the pooled effect estimates. To avoid biased results, a meta-analysis should always be a product of a foreseeing systematic literature review based on a detailed *a priori* established study protocol.

The main objective of a systematic literature search is to aim to identify all relevant studies using several electronic databases and an additional complimentary manual search. Nonetheless, finding a balance between feasibility in terms of time efficiency without jeopardizing missing out on important publications is challenging. Theoretically, there is always a possibility that not all relevant papers were captured by the search. Furthermore, the *a priori* defined hierarchy for inclusion and exclusion criteria can favourably be established by applying the PICOS' model, where "P" stands for population, "I" for intervention, "C" for the comparator, "O" for an outcome, and "S" for study design.(146) Occasionally, additional criteria are set, such as time period, publication language, or geographical area. However, each additional restriction may introduce selection and influence the generalisability of the pooled effect estimates. In Study III, no additional criteria were set for the search, and colleagues translated studies published in foreign languages.

When the outcome is rare, standardized risk estimates can be pooled together in a meta-analysis providing pooled effect sizes with conventional 95% confidence intervals. Each included study is assigned a weight, which is influenced by sample size and confidence intervals. Thus, larger studies may potentially influence the results to a greater extent as compared with smaller studies, although the effect of weights is less prominent in random-effects models. Beyond the traditional meta-analytic approach, another interesting dimension for dose-response analyses is whether departure from linearity is present. In brief, a non-linear dose-response meta-analysis is conducted by i) fitting a dose-response model within each publication, ii) pooling the publication-specific effect estimates together, iii) testing for non-linearity using a statistical significance test, and iv) lastly graphing the association.(135) I would argue that in such an approach, the most challenging step is to find a justifiable way to quantify the exposure, as in reality, the included publications are likely to classify the exposure in different ways. In Study III, the exposure to antibiotics was quantified based on a median value of each exposure category.

Random-effects models assume between-study heterogeneity and take into account random error within each study. When the exposure is complex, it is expectable that the included publications do not measure the same parameter, which is one of the cornerstone assumptions of fixed-effects models. Therefore, random-effects models are preferable when the heterogeneity is higher, as these models assume in between publications heterogeneity in addition to random error.(164)

Clinical and statistical heterogeneity might be influenced by several factors, including clinical settings (for example, treatment type and duration), study design, population, and follow-up time. Therefore, higher heterogeneity is expectable, especially if the exposure is as complex as in Study III. To assess statistical heterogeneity between the included publications Cochrane's Q-test and Higgins's I^2 test can be applied. However, the I^2 test is preferable since the Q-test has a limited ability to detect heterogeneity in smaller studies with low power.(164, 165) Another possibility to explore heterogeneity is to perform various sensitivity analyses, as was done in Study III. To address potential sources of heterogeneity, a meta-regression could be an option, given a reasonable number of publications can be included.(165)

Furthermore, papers reporting statistically significant results might be more likely to be accepted for publication, possibly leading to an overestimation of the effect. Funnel plots allow us to inspect the presence of a potential publication bias visually, and an asymmetrical distribution could be an indication of this. However, the word publication bias is rather strong, and perhaps small study effects would be a more appropriate description of the measure.(166) To complement the visual assessment of funnel plots, Egger's test estimates the potential asymmetry utilizing statistical significance testing. Yet, the test has limited value when less than ten publications are included.(136, 165)

Several quality assessment tools may be applied simultaneously,(133, 134) and even carefully tailored for a better fit as shown in **Figure 12**.

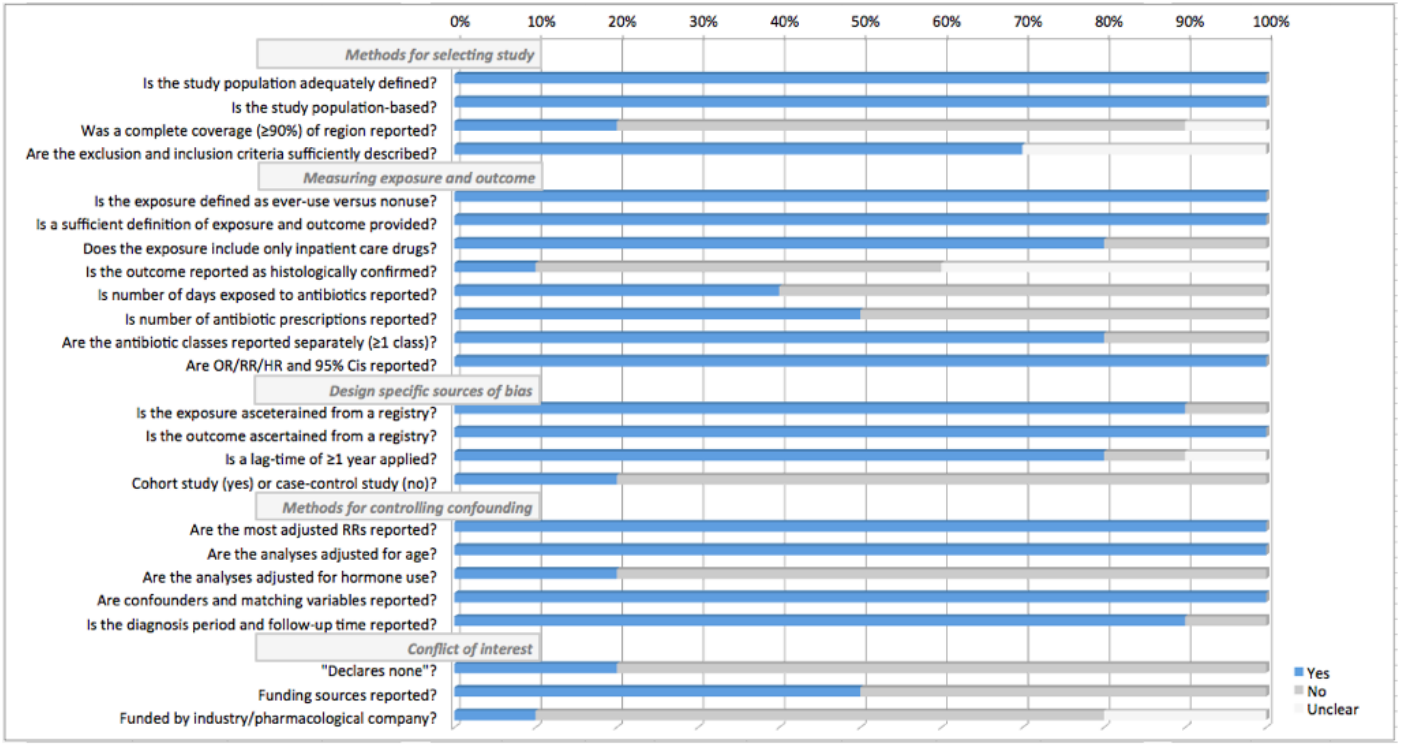

Figure 12*. A customised quality assessment tool applied in Study III. The x-axis presents the considered quality variables, and the y-axis provides the percentages fulfilling the variables presented on the x-axis. Thus, 100% indicates that all of the studies fulfilled the criteria given on the x-axis. Abbreviations: OR: odds ratio, RR: relative risk, HR: hazard ratio, 95% CI: 95% confidence interval.*

Nonetheless, the value of quality assessment tools should not be overestimated. Whilst these tools may provide valuable information to identify publications that differ from others, they are not entirely objective. Therefore, publications should not be excluded based on lower-quality assessment scoring alone. Instead, a more informative solution would be to conduct sensitivity analyses, for example excluding studies with the lowest quality scores.

7 DISCUSSION

7.1 THE MAIN FINDINGS

This thesis within clinical epidemiology has investigated the carcinogenic risks associated with long-term exposure to menopausal hormone therapy and oral antibiotic treatment – both pharmacological treatments which either include oestrogens or may modulate the metabolism of oestrogens. Three of the theses included publications focused on investigating the cancer risk among users of menopausal hormone therapy, which is the primary treatment for vasomotor symptoms experienced by half up to 80% of women during menopause worldwide. One of the publications explored the posed link between exposure to oral antibiotics and excess colorectal cancer risk, as the high antibiotic consumption and increasing burden of cancer underscore our need to consider and investigate all potential risk factors.

We have shown that the cancer risk associated with systemic menopausal hormone therapy is seemingly overestimated, given the total cancer risk was only slightly increased. The excess risk was mainly related to female reproductive organ cancers. Per contra, MHT appeared to reduce the risk of gastrointestinal tract cancers, and our data indicated that prediagnostic MHT use could even improve survival from colorectal cancer. Overall, MHT did not appear to increase the cancer risk if initiated at close proximity to menopausal onset among women without contraindications or individuals without an increased risk of breast, ovarian or endometrial cancer. Whereas we can see that the number of MHT prescriptions has remained rather stable during the past decade, a deep drop by over 30% was noted after the millennium following the Women's Health Trial's results. This decrease in MHT prescriptions could be explained at least partly due to fear of carcinogenic effects. However, our results indicate that the fear of cancer risk is overestimated among otherwise healthy women who initiate treatment before 60 years of age.

Furthermore, we found a link between oral antibiotic intake with an elevated colorectal cancer risk. This association was noted particularly among broad-spectrum antibiotic users, whilst no association was shown among individuals exposed to narrow-spectrum antibiotics. Moreover, the evidence for the non-linear dose-response relationship was weak, and the found association may not necessarily be of causal nature. However, together with the widespread pandemic of antibiotic overconsumption, these findings warrant more strict antibiotic stewardship.

7.2 STUDIES ON MENOPAUSAL HORMONE THERAPY

7.2.1 Study I

In Study I, we have shown a slightly (9%) increased total risk of cancer among contemporary users of systemic menopausal hormones, compared with the background population. Whereas the excess risk was mainly associated with typical female reproductive organ cancers, the overall risk of cancer was almost counterbalanced by the reduced risk of all gastrointestinal tract cancers. Altogether, the association appeared to be stronger among women receiving EP-MHT treatment than women who received oestrogen-only therapy, yet the association was inconsistent across the 16 different cancer sites and ages. For EP-MHT users, the excess risk was associated with mainly continuously administered regimens. Notably, the highest excess risk was found among the most prescribed EP-MHT regimens, namely continuously administered testosterone-derived combination therapy regimen.

Whilst population-level studies on the total cancer risk were relatively non-existent, our findings are in line with earlier studies suggesting an increased risk of particularly breast cancer, associated primarily with EP-MHT use (7, 10, 104, 167). Our findings support the potential role of oestrogens in reducing the risk of sex hormone-related cancers.(12, 15, 168, 169) In comparison to the Women's Health Trial in 2002, including women 50-79 years old at the baseline (mean age 64 years), our results showed that the risk of breast cancer changes by age at treatment initiation,(9) supporting the current clinical guidelines recommending treatment start before the age of 60 years.(90, 91) Moreover, compared to our cohort, the trial participants were substantially heavier (≈45% of the participants who underwent hysterectomy had body mass index over 30 kg/m^2), and thus the results could reflect a cumulative effect of endogenous and exogenous oestrogens.(9, 170) It has also been suggested that obese women may express more severe vasomotor symptoms, although underlying mechanisms are not well understood.(74) In general, as compared with a trial design, this present study included both prevalent and incident users of MHT. However, this composition reflects a real-world mixture of MHT users.

In summary, contemporary use of systemic MHT treatment was linked with a slightly elevated total risk of cancer. However, the risk varied by women's age at the start of the treatment and the various MHT treatment options. The cancer risk appeared to be minimal if treatment was initiated close to menopausal onset among women without contraindications or high risk of breast, ovarian

or endometrial cancer. Nonetheless, a thorough individual risk-benefit assessment should be conducted before treatment initiation, and other aspects than the potentially carcinogenic or chemopreventive effects should be carefully considered.

7.2.2 Study II

In study II we have shown that women receiving particularly current EP-MHT treatment were at a 38% increased risk of primary ovarian cancer compared with women not receiving menopausal hormones, whilst only a marginal association was noted among past EP-MHT users. In a subgroup analysis among EP-MHT receivers, the most prescribed continuous testosterone-derived regimens were linked to a 50% increased ovarian cancer risk compared with MHT non-users, whereas a marginal association was shown among continuous progestin-derived regimens. Notably, no clear association was found for sequential regimes. Among E-MHT only users, a strong inverse relationship with ovarian cancer was observed, yet this association requires thorough further clarification.

A previous meta-analysis based on population-based studies found a 22% increased ovarian cancer risk among E-MHT only users.(106) One possible explanation for this discrepancy could be that tubal ligations and oophorectomies could be underreported in Sweden, particularly among the older women, as the Patient Registry became nationwide in 1987. The fact that the here found inverse association did not vary across different oestrogen formulations, age at initiation or current versus past use argues for the possibility of underreported oophorectomies. The same meta-analysis suggested a 10% lower risk among users of EP-MHT as compared to E-MHT users,(106) whilst an individual participant meta-analysis including 52 studies indicated a 40% elevated overall risk among MHT users.(105) In this present study, EP-MHT users had a 17% increased risk of ovarian cancer. Whereas induction of MHT treatment is contraindicated among women with unknown vaginal bleeding,(91, 151, 152) the potential risk of reverse causality cannot be excluded. Consequently, the shown increased risk among EP-MHT users could partly be explained by symptoms as of yet undiagnosed ovarian cancer mistaken as menopausal symptoms, selectively among women with intact uterus or ovaries,(153, 171) potentially leading to overestimation of the risk among this group of women.

In conclusion, especially current use of combination therapy may increase ovarian cancer risk, whilst the association with unopposed oestrogen-only treatment remain unclear.

7.2.3 Study IV

This nationwide cohort study found over 30% lower colorectal cancer-specific and all-cause mortality risk among women who had received systemic menopausal hormones before their colorectal cancer diagnosis. The findings indicate that past use of E-MHT may improve colorectal cancer survival, and thus prediagnostic oestrogen-only users may have a more favourable prognostic outlook for colorectal cancer, compared with MHT non-users. However, current E-MHT use was associated with elevated all-cause mortality risk, especially among women diagnosed at 70 years or later. Furthermore, current use of combination therapy among women aged 60-69 years suggested an increased risk of colorectal cancer.

In a previous Swedish study, no association was found for post-diagnostic use of EP-MHT.(68) The discrepancy between our results could potentially be explained by healthy user bias. It is possible that particularly post-diagnostic combination therapy could be favoured among healthier women given the association with especially breast cancer. (7, 9, 77) Overall, our findings are similar to the results of a screening trial and a recent meta-analysis, both showing a reduced colorectal cancer-specific and all-cause mortality risk among MHT user.(70, 172) However, the meta-analysis, which included five cohort studies, found risk reduction among current MHT users.(172) Here, we observed a lower risk of colorectal cancer among past users of E-MHT. This discrepancy could be explained by different definitions of current versus past use. Our findings are biologically plausible, as it is likely that past users have been exposed to oestrogens for a longer duration of time, and it could be expected that the potentially protective effect of oestrogens would take time until it is shown. Moreover, we observed an increased risk of especially all-cause mortality among current E-MHT users 70 years or older. Whereas it is possible that the cell-proliferative properties of oestrogens could contribute to this increase,(173, 174) potential reverse causality cannot be ruled out (*id est* these women may have had an underlying condition warranting MHT treatment).

In conclusion, particularly past exposure to E-MHT only treatment may improve survival from colorectal cancer. However, the association with mortality overall varied by the different MHT types, age, and current versus past use. Furthermore, whereas the results of this study indicate that oestrogen-only therapy may have a beneficial effect, it should be considered that menopausal hormones are associated with other deleterious health effects such as an elevated risk of other cancer types and cardiovascular events.(8, 10, 85) Therefore, systemic MHT may not be a suitable

treatment option for all women, particularly for those at high risk for breast, ovarian or endometrial cancer.

7.3 STUDY ON ORAL ANTIBIOTIC TREATMENT

7.3.1 Study III

This extensive systematic review and dose-response meta-analysis found a link between oral antibiotic treatment and colorectal cancer risk, with a modestly increased risk among individuals exposed to antibiotics. Notably, the excess risk of cancer was associated mainly with broad-spectrum antibiotics, whereas narrow-spectrum antibiotics did not suggest any link. Furthermore, the dose-response analyses did not reveal any more substantial proof for a potential dose-response relationship, and risk patterns' shapes were comparable within the colorectal continuum. However, the found positive association between oral antibiotic use and an increased colorectal cancer risk should be interpreted cautiously because this complex association may not necessarily reflect a causal relationship.

7.3.1.1 Strengths and limitations

This study's main strength is the underlying meticulous, systematic literature review and the novel *state-of-art* methods exploring the shape of the posed risk pattern. The meta-analysis included in total more than four million unique individuals and over 73,550 people who had been diagnosed with colorectal cancer, increasing the statistical power of the study and enhancing the generalisability of the results. As compared with other dose-response meta-analyses, one of the significant advantages of this present analysis was the quantification of each exposure category based on the median values since a binary categorization to low versus high dose could lead to loss of important data.

The main limitation of this study is the inability to draw an inference of causality, and the role of confounding by indication cannot be eliminated. The shown weak dose-response pattern could indicate confounding by indication, as indications requiring higher doses and longer duration of antibiotic use may indicate a chronic underlying disease rather than antibiotics causing cancer. The data for the dose-response relationship could be expected to be more reliable among individuals with lower cumulative doses, as it is likely that fewer individuals are exposed to higher doses of antibiotics. However, a link between antibiotic use and lung cancer has been suggested. For this

association, confounding by incitation could be a more significant concern, given we could expect higher cumulative use of antibiotic treatment for infections of the respiratory tract.(18, 175)

Another intriguing hypothesis is the role of the gut microbiome,(120, 122, 176) underscoring the need for more mechanistic studies aiming to understand the drug-microbiome interactions on the development of colorectal cancer. The here shown differences between broad- and narrow-spectrum antibiotics could argue for the role of antibiotic use related dysbiosis. Our findings are in line with a recent Swedish nested case-control study indicating a more pronounced colorectal cancer risk among broad-spectrum antibiotic users (OR= 1.23, 95 % CI 1.18-1.29) than narrow-spectrum antibiotic users (OR=1.05, 95% CI 1.01-1.10).(177) Thus, the here shown association of oral antibiotics with increased colorectal cancer risk may not be causal, and it could indicate a possible role of dysbiosis in carcinogenesis.

Nonetheless, the funnel plot was not visually symmetrical, indicating a possible presence of small-study effects (**Figure 13**).

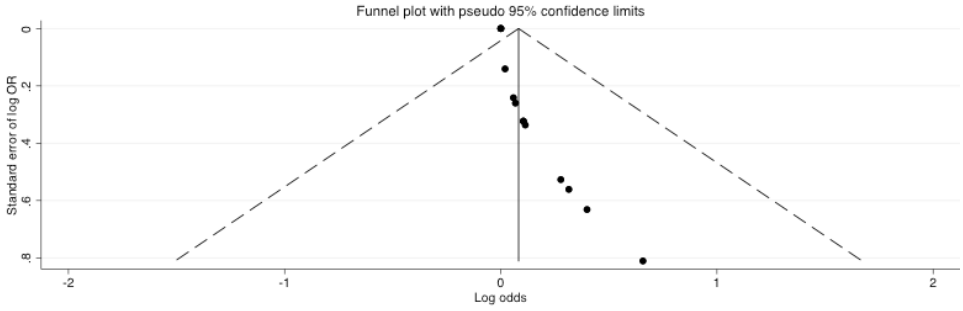

Figure 13. *The funnel of the ten included publications in Study III investigating the antibiotic use associated risk of colorectal cancer.*

Furthermore, the pooled risk estimates' comparability from the original publications might have been undermined by the different periods of exposure ascertainment between the included studies. Whereas most of the publications included in the meta-analysis had comparable exposure periods, beginning from the 1990s, the dose and formulations might have changed over time. Therefore, it should be kept in mind that the pooled effect sizes reflect a mixture of various exposures and periods of exposure.

In conclusion, exposure to oral antibiotics was associated with elevated colorectal cancer risk, yet the shown non-linear dose-response relationship was weak, and alternative hypotheses could explain the found association.

8 CONCLUSIONS

- Menopausal hormone therapy is associated only with a slightly increased total risk of cancer; however, the risk varies by age at treatment start and the various MHT treatment options.

- Combination therapy is seemingly associated with increased ovarian cancer risk, whereas oestrogen-only therapy did not appear to increase the cancer risk. Although less frequently prescribed, transdermal menopausal hormones might involve a lower cancer risk than orally administered treatment.

- Past use of oestrogen-only therapy before colorectal cancer diagnosis may improve survival from colorectal cancer.

- Exposure to oral antibiotics is linked to increased colorectal cancer risk, yet with weak evidence for a non-linear dose-response relationship, and the here shown association is not necessarily causal.

9 POINTS OF PERSPECTIVE

This thesis aimed to assess the long-term effects of two commonly prescribed drugs on cancer risk and colorectal cancer mortality. The included nationwide, population-level registry-based studies provided robust information that can aid in clinical risk assessment. The overall cancer risk among ever-users of MHT was shown to be weak, and MHT use does not appear to increase the cancer risk when initiated near to menopausal onset among otherwise healthy women without contraindications or a high risk of, especially breast, ovarian or endometrial cancer. However, the effect of MHT use on all-cause mortality is highly relevant when considering whether MHT treatment is harmful. Per contra, the association with mortality could differ from the cancer incidence since some evidence indicates that MHT associated tumours might have a more favourable prognostic outlook.

Another interesting aspect, which has already been discussed in an international context, would be to investigate whether a combination of intrauterine progestin and orally or transdermally administered oestrogen therapy could potentially provide a safer treatment option compared with the currently prescribed oral EP-MHT treatment for women with an intact uterus. Here, our results suggested, although not confirmed, that transdermally administered systemic MHT may involve a lower ovarian cancer risk than oral preparations. Therefore, it would be highly informative to explore this potential association for other outcomes, aiming to identify safer treatment options with possibly lower risk of cancer and other deleterious health effects.

Furthermore, the shown link of oral antibiotic use with an increased colorectal cancer risk perhaps raises more questions than the study can answer. Whereas the found association is not necessarily causal, the widespread pandemic of antibiotic overuse and the following subsequent problem with new emerging pathogens and antibiotic resistance underscores the need for more in-depth mechanistic studies. Considering antibiotics may not cause cancer per se, but the association could be mediated by the gut microbiome, this hypothesis warrants studies on potential drug-microbiome interactions, especially for diseases associated with a high cumulative intake of antibiotics.

10 POPULÄR VETENSKAPLIG SAMMANFATTNING

Denna doktorsavhandling syftade till att kartlägga de olika långtidseffekterna av hormonbehandling i klimakteriet (HT) och vanligen förskrivna antibiotika – båda utbredda farmakologiska behandlingar, som antingen innehåller estrogener eller kan förändra metabolismen av estrogener. Tre av de inkluderade publikationerna baserades på omfattande rikstäckande och befolkningsbaserade kohortstudier, syftandes på att utvärdera cancerrisken bland alla kvinnor som erhållit HT-behandling under studietiden. Hormonbehandling är den primära behandlingen för vasomotoriska symptom vid klimakteriet och dessa upplevs av 50 – 80 % av kvinnorna över hela världen. En av de inkluderade publikationerna fokuserade på antibiotikabehandlingar med ändamålet att undersöka det associerade sambandet mellan antibiotika med kolorektal cancerrisk. Detta eftersom den utbredda användningen av sedvanliga antibiotika och den ökande cancerbördan lyfter fram behovet att systematiskt utforska alla potentiella riskfaktorer.

Denna doktorsavhandling är unik på grund av dess tre kohortstudier med riksomfattande och befolkningsbaserade tillvägagångsätt, baserade på de nationella svenska hög-kvalitativa registren. Därtill, inkluderades en stor systematisk litteratursökning och meta-analys som tillämpande "state-of-art" metoder.

Vi har påvisat att cancerrisken associerad med systemisk hormonbehandling till synes överskattas, apropå den totala nettoeffekten av HT-behandlingen på cancerutveckling var lindrig. Den förhöjda cancerrisken var främst associerad med bröst-, äggstocks- och livmodercancer hos kvinnor som inledde behandlingen i äldre ålder. Däremot fann vi indikationer för en reducerad risk av gastrointestinala cancrar bland HT-användare och våra data tyder på att prediagnostisk användning av estrogener potentiellt skulle kunna även förbättra överlevnaden från kolorektal cancer. Dessa resultat skulle möjligen kunna stödja den kemopreventiva rollen av estrogener associerade med könshormonrelaterade cancerformer.

Medan vi har märkt att antalet utskrivna HT-recept har stannat på en relativt stabil nivå under det senaste decenniet, minskade antalet recept med över 30 % i Sverige efter millenium följandes kliniska resultat tydandes på en högre bröstcancerrisk hos kvinnor exponerade till hormonbehandling. Denna kraftiga nedgång i mängden av utskrivna HT-recept kan delvis

förklaras av rädsla för hormonbehandling associerade carcinogena effekter. Våra resultat tyder dock på att menopausala hormoner inte ökar cancerrisken nämnvärt om behandlingen initieras i nära anslutning till klimakteriets början hos annars friska kvinnor utan kontraindikationer eller en ökad risk för bröst-, äggstocks-, eller livmodercancer.

Därtill upptäckte vi en koppling mellan exponering till vanligen förskrivna antibiotika och en ökad risk av kolorektal cancer, som var kopplad särskilt till bredspektrumantibiotika. Emellertid var bevis för dos-respons förhållandet svagt och den påvisade sambanden kanske inte är orsaksrelaterad. Icke desto mindre, tillsammans med den utbredda överanvändningen av antibiotika, motiverar dessa resultat ett striktare förhållningssätt till utskrivning av antibiotika.

Avslutningsvis, denna avhandling möjliggjorde kartläggandet av cancerrisken sammankopplad till vanligen förskrivna läkemedel associerade med det kvinnliga könshormonet estrogen. Våra resultat antyder att estrogener skulle kunna minska risken av de typiskt mansdominerande gastrointestinala cancrar och eventuellt även förbättra överlevnaden från könshormonrelaterade cancrar. De olika tillvägagångssätten som använts i de olika delstudierna försäkrade en noggrann utvärdering från ett flertal olika aspekter. Å andra sidan, den uppmärksammade associationen med vanliga antibiotika skulle kunna förklaras med alternativa hypoteser – och i synnerhet den möjliga rollen av dysbiosis bör efterforskas grundligt.

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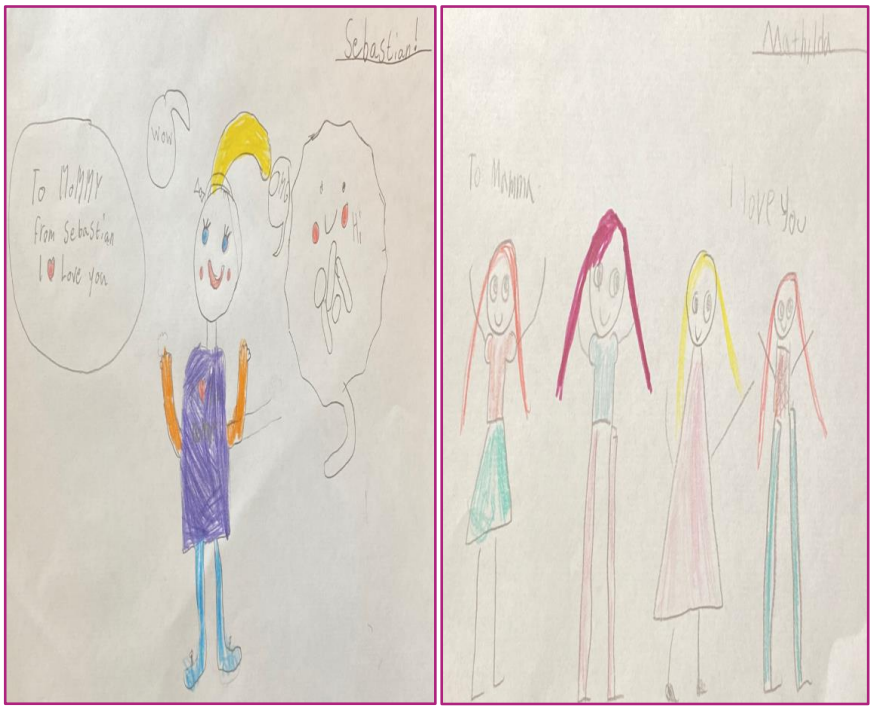

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